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Regulation of goldfish macrophage development *in vitro*

by

Daniel R. Barreda ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Physiology and Cell Biology

Department of Biological Sciences

Edmonton, Alberta
Fall 2003

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, the thesis entitled: Regulation of goldfish macrophage development *in vitro*, submitted by Daniel R. Barreda in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology and Cell Biology.

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To my parents,
for everything I know about dedication
I learned from them.

ABSTRACT

Over 100 years after the first description of macrophages by Metchnikoff, there are still questions as to the mechanisms leading to the heterogeneity of their lineage. Current views are based on the mononuclear phagocyte system (MPS) theory, where all macrophages are derived from circulating blood monocytes and ultimately from hematopoietic stem cells in the bone marrow. My studies on the regulation of fish macrophage development suggested that alternate pathways of macrophage hematopoiesis contribute to this heterogeneity.

Characterization of primary kidney macrophage (PKM) cultures from goldfish hematopoietic tissues indicated that three distinct subpopulations developed in response to endogenous macrophage growth factors. Morphological, cytochemical, flow cytometric, and functional characterization of cultured macrophages indicated that these subpopulations corresponded to three distinct stages of macrophage differentiation: progenitors, monocytes, and "mature" macrophages. These macrophage subpopulations also expressed several differentiation markers including the hematopoietic stem cell antigen AC133, c-kit, CD63, macrosialin, c/EBPβ, legumain, and the colony stimulating factor receptor-1 (CSF-1R). There appeared to be a stringent control between those early progenitors that self-renewed, and those that were recruited into the maturation pathways. Furthermore, it was found that upon commitment, cells developed through two distinct differentiation pathways: one consistent with the "classical" pathway of macrophage development (progenitors → monocytes → mature macrophages), and an "alternate"

pathway where mature macrophages appeared to rapidly develop from early progenitors in the absence of an intermediate monocyte stage.

Macrophages developing through the "alternate pathway" (AP-mϕ) represented a unique subset of spontaneously growing cells. Their self-renewal was promoted by endogenous macrophage growth factors (MGF), and effectively controlled through the production of endogenous macrophage growth inhibitory factors (MGIF). Characterization of these MGIF identified a novel soluble form of the CSF-1 receptor. The discovery of sCSF-1R and other positive and negative regulators of fish macrophage development, through a differential cross-screen approach, highlighted the inherent complexity in the hematopoietic regulatory machinery of lower vertebrates. Moreover, characterization of the alternate pathway of macrophage development suggested the existence of evolutionarily-conserved mechanisms contributing to the generation of macrophage diversity.

ACKNOWLEDGEMENTS

The work presented in this thesis is truly a reflection of the tremendous support that I have received over the last few years. As such, I am indebted to several friends and colleagues who have contributed in one way or another to my growth as a researcher. I am especially thankful to my supervisor, Dr. Miodrag Belosevic, for his continued support and guidance. I am grateful to my supervisory committee, Dr. Larry Guilbert and Dr. Norman Stacey, for sharing their experience and excitement for science. I would also like to thank Dr. Paul Wong, Dr. Kathy Magor, and Dr. Brad Magor for their time and valuable insights into the world of molecular biology. I also extend thanks to Dr. Bill Samuel, Dr. Laura Frost, Dr. Allen Shostak, Dr. Greg Goss, Dr. Jeff Goldberg, and Dr. John Chang for their help and support. I am also thankful to Dr. John Samuel and Dr. Niels Bols for their careful analysis of this thesis.

Many thanks to my fellow graduate students Dr. Norman Neumann, Dr. James Stafford, Dr. Dianne Bienek, Ms. Debbie Plouffe, Ms. Kristine Scott, Mr. Patrick Hanington, Ms. Michelle Patterson, and Mr. Micah Chrenek for their friendship, collaborations, and many scientific discussions.

I am grateful to the Natural Sciences and Engineering Research Council of Canada, the Department of Fisheries and Oceans Canada, the Department of Biological Sciences, and the University of Alberta for their financial support.

Very special thanks for the people that really made this degree memorable: Mr. Mathew Bryman, Dr. David Bressler, Mr. Douglas McFarlane, Dr. Brian Weiss, Ms. Jadwiga Stepczynski, Mr. Luis Hidalgo, Mr. Jay Varghese, the TAPS and Immunet crews, and many other friends throughout the Department of Biological Sciences and the Department of Medical Microbiology and Immunology.

Most importantly, I would like to thank Alayne and the rest of my family for their continued love, support, and encouragement. All that I have achieved is better because I can share it with you.

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LIST OF ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
AP-mϕ	alternate pathway macrophage
AREs	AU-rich elements
BLAST	Basic Local Alignment Search Tool
bp	base pair
CCM	cell conditioned medium
cDNA	complementary DNA
C/EBP	CCAAT/enhancer binding protein
CFU	colony forming unit
CFU-GM	common bipotent granulocyte-macrophage progenitor
CMP	common myeloid progenitor
CNPase	2', 3'-cyclic-nucleotide 3'-phosphodiesterase
ConA	concanavalin A
CoQ	co-enzyme Q
CRH	cytokine receptor homology
CSF	colony stimulating factor
CSF-1R	colony stimulating factor-1 receptor
DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
D-PBS	Dulbecco's phosphate-buffered saline
DPBS-G	Dulbecco's phosphate buffered saline with glucose
FPLC	fast performance liquid chromatography
Epo	erythropoietin
EtBr	ethidium bromide
FACS	fluorescence activated cell sorter
FGF	fibroblast growth factor
Flt	fms-like tyrosine kinase
Fms	feline sarcoma virus

FN III	fibronectin type III
G-CSF	granulocyte colony-stimulating factor
GH	growth hormone
GMCL	goldfish macrophage cell line
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP-FPLC	gel permeation fast performance liquid chromatography
HBSS	Hank's balanced salt solution
HSC	hematopoietic stem cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
JAK	janus kinase
KL-derived CCM	kidney leukocyte-derived cell conditioned medium
Kd	dissociation constant
kDa	kilodalton
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
Mϕ	macrophage
M1 cells	mouse myeloid leukemia cell line
MAF	macrophage activating factor
MAPK	mitogen activated protein kinase
M-CSF	macrophage colony stimulating factor
mCSF-1R	membrane bound CSF-1 receptor
me	motheaten
MGF	macrophage growth factor
MGIF	macrophage growth inhibitory factor
MHC	major histocompatibility complex
Mona	monocytic adaptor
MPS	mononuclear phagocyte system
M_r	molecular weight

mRNA	messenger RNA
NBT	nitroblue tetrazolium
NO	nitric oxide
PFU	plaque forming unit
P.I.	proliferation index
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PHA	phytohemagglutinin
PI3K	phosphatidylinositol 3-kinase
PKM	primary kidney macrophage
PMA	phorbol myristate acetate
PMS	phenazine methosulfate
Poly-A	poly-adenylation
PTPase	phosphotyrosine protein phosphatase
RACE	rapid amplification of cDNA ends
RICH	regeneration induced CNPase homolog
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase PCR
sCSF-1R	soluble form of the CSF-1 receptor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src-homology 2
SHIP	Src-homology 2-containing inositol phosphatase
SHP-1	Src-homology-2-domain-containing protein tyrosine phosphatase 1
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
SUMO-1	small ubiquitin-like modifier-1

TGF	transforming growth factor
TMS	tricaine methanesulfonate
TNF	tumor necrosis factor
Tpo	Thrombopoietin
UTR	untranslated region
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

CHAPTER I: INTRODUCTION AND LITERATURE REVIEW [1]

1. INTRODUCTION

Macrophages are essential to host defenses and to the maintenance of homeostasis. Macrophages: a) defend against microorganisms and tumor cells, through phagocytosis and the activation of cytotoxic killing responses; b) regulate inflammatory responses, through the secretion of mediators and complement; c) initiate acquired immune responses, through antigen presentation and activation of T-lymphocytes; d) regulate hematopoiesis through the production of colony-stimulating factors and other cytokines; and e) are central to wound healing processes (1-3). They are ubiquitously distributed within the vertebrate body, and macrophages or macrophage-like cells have been described in organisms ranging from sea sponges and echinoderms to higher vertebrates.

Macrophage development from pluripotent progenitors, or hematopoiesis, is under the stringent control from paracrine, juxtacrine, and autocrine cytokine mediators. Of these, the colony stimulating factors (CSFs) have been identified as key regulators at all stages of macrophage differentiation and have proved central to the understanding of the regulatory pathways modulating their development. On the other hand, little is known about the mechanisms leading to the generation of functionally mature macrophages in organisms outside mammalian systems, and the role that these effector cells play in host defense mechanisms and the maintenance of biological integrity.

In the first chapter of this thesis, I review some of the most salient information on CSF biology and myeloid development collected over the last three decades. In particular, I focus on the regulation of macrophage development and function by the colony stimulating factors in the context of a complex network of regulatory pathways that are carefully integrated at the level of gene expression and culminate in an overall hematopoietic response. Chapter II contains a detailed description of the materials and methods used throughout this thesis project. In Chapter III, I report on the generation of goldfish macrophages *in vitro* in response to endogenous macrophage growth factors. In this chapter, I describe the *in vitro* cultivation techniques used in the establishment of macrophage cultures. In addition, I characterize the developing macrophages using morphological, cytochemical, flow cytometric, and functional analyses. In Chapter IV, I report on the detailed characterization of the proliferation events and differentiation shifts leading to the generation of mature macrophages from early progenitors using a fluorescent membrane dye, PKH26, and flow cytometry, and show that there appears to be a temporal component governing fish macrophage development. In Chapter V, I describe the kinetics of fish macrophage growth and show that the production of putative endogenous macrophage growth factors (MGF) and growth-inhibiting factors (MGIF) appear to be differentially regulated in different phases of macrophage development. In Chapter VI, I describe the characterization of a novel negative regulator of macrophage hematopoiesis, the soluble form of the CSF-1 receptor. I show the molecular identification, cloning, protein expression, and functional characterization of this naturally occurring transcript and discuss its relevance in the context of macrophage hematopoiesis control. In Chapter VII, I document the identification of several additional potential hematopoietic regulators by using a differential cross-screen molecular approach. This was aimed at providing a more global understanding of the MGF-mediated hematopoiesis regulation in the context of expression analysis for a broad spectrum of genes. The final chapter, Chapter VIII, is a general discussion of the findings reported in this thesis. I developed a new model for the generation of macrophage hematopoiesis in the adult fish, and considered the evolutionary conservation of such mechanisms in mammalian systems. Furthermore, I suggested the possible routes to be taken in subsequent studies based on our recent findings and the various reagents that have been effectively generated throughout the course of my Ph.D. thesis.

---

[1] A version of this chapter has been published:

Barreda, Hanington, and Belosevic (2003) *Dev. Comp. Immunol.* In Press.

D.R. Barreda was involved in the data collection, analysis, and writing of the manuscript.

2. OBJECTIVES OF THE THESIS

The main objective of my thesis was to examine the mechanisms involved in the regulation of fish macrophage development. The characterization of these mechanisms will facilitate the design of prevention strategies against fish pathogens and provide an improved understanding of the evolutionary conservation of macrophage-derived defense mechanisms and their overall function. Hence, the specific aims of my research project were:

1. To functionally characterize the development of fish macrophages *in vitro*.
2. To identify positive and negative modulators of fish macrophage hematopoiesis.
3. To identify genes that are differentially expressed in developing fish macrophages *in vitro*.
4. To characterize the mechanisms regulating teleost macrophage hematopoietic events.

3. LITERATURE REVIEW: REGULATION OF MYELOID DEVELOPMENT AND FUNCTION BY THE COLONY STIMULATING FACTORS

3.1. Introduction

Hematopoiesis refers to the formation and development of mature blood cells resulting from the survival, proliferation, commitment and differentiation of early progenitors (4, 5). It provides a mechanism by which cells that have expired or are damaged can be replaced through normal 'steady state' cellular turnover. Up-regulation of the hemopoietic machinery during stress conditions is critical to the establishment of proper defenses against invading pathogens and the maintenance of homeostasis. Hematopoietic proliferation, commitment, differentiation, and survival events are coordinated through interactions with cells and extracellular matrix components of the surrounding microenvironment, through interactions with soluble factors that are produced locally or arrive from the circulation, as well as through intrinsic mechanisms in part regulated by stochastic events. It is the integration of these extracellular and intracellular stimuli, through an intricate network of complex but finely tuned regulatory pathways that culminates in an overall response and the expression of specific genes.

Early in the development of a blood cell, pluripotent stem cells reside within specialized niches in hematopoietic tissues like the bone marrow or head kidney in teleosts. These cells are in a quiescent state until they receive signals aimed at the up-regulation of hematopoietic events. There are over 20 soluble hematopoietic regulators known to arrive from circulation or act on pluripotent stem cells in a paracrine, juxtacrine, or autocrine manner (6, 7). Of these, the colony-stimulating factors have been widely studied for over 30 years and have proven essential to the development of cells of the eight blood lineages. They include the macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and the multi colony-stimulating factor better known as interleukin-3 (multi-CSF or IL-3). These factors show both functional pleiotropy, exhibiting a wide variety of biological functions on various tissues and cells, as well as significant redundancy, being able to exert similar and overlapping functions on specific cells (8). Overall, the CSFs mediate the survival, proliferation, differentiation, and functional modulation (chemotaxis, degranulation, activation, adhesion, cytotoxicity, mRNA phenotype changes) of various populations of mature blood cells and their precursors (9-12).

In this review we focused on colony-stimulating factors, and their role in the maintenance of immune competence through the conservation of adequate blood cell numbers. In particular, we examined the CSF-mediated regulation of myeloid cell development since the CSFs play a central role in their development from early hematopoietic progenitors to mature effector cells of the immune system. The findings of the last 30 plus years which have guided CSF research are discussed; from early colony-forming assays, testing growth factor activity from cell and organ culture conditioned media, to their current widespread applications as therapeutics in the control of immune responses against disease. We considered the structural characteristics of CSFs and their receptors, and examined the main determinants behind the modulation of their gene expression patterns. We explored the reasons behind the significant functional redundancy exhibited by the CSFs, as well as the mechanisms responsible for their specificity. Most of this work was based on information on CSFs from human and mouse species. However, an attempt was made to integrate recent findings in other systems in order to highlight a more widespread role for CSFs throughout evolution.

3.2. Macrophage colony-stimulating factor (M-CSF)

The macrophage colony-stimulating factor (M-CSF or CSF-1) was the first of this group of growth factors to be purified (13). It has also been formerly referred to as macrophage growth factor (MGF) (14) or macrophage and granulocyte inducer IM (MGI-IM) (15). This homodimeric sialoglycoprotein is the principal regulator of the survival, proliferation, and differentiation of macrophages and their precursors (16, 17), and also synergizes with other cytokines to mediate the proliferation of early hematopoietic progenitors (18-20). It was originally found in murine serum, human urine, and cell-conditioned medium from murine fibroblast L cell line (13, 21, 22). It was also identified in extracts from a variety of tissues, and the supernatants from various cell and organ cultures (23, 24). Maximum stimulation of M-CSF-mediated proliferation can be observed at concentrations of 250 pM, and in the case of bone-marrow colony-forming cells, proliferation can be detected at concentrations as low as 1 pM (24). The normal range for serum CSF-1 concentration is between 150-500 U/ml or 3-8 ng/ml (18, 25-28).

M-CSF is synthesized by a variety of cell types including endothelial cells, fibroblasts, bone marrow stromal cells, osteoblasts, thymic epithelial cells, keratinocytes, astrocytes, myoblasts, mesothelial cells, endometrial gland cells, and the placenta-trophoblast decidual stroma [reviewed in (29-31)]. Stimulation of M-CSF production can also be achieved following activation of cells including monocytes, macrophages, microglial cells, endothelial cells, T lymphocytes, B lymphocytes, fibroblasts, chondrocytes, and mesangial cells. It is interesting to note that a variety of inflammatory cytokines can up-regulate mononuclear phagocyte expression of M-CSF including GM-CSF (9), TNF-α (32), IL-1 (33-35), and IFN-γ (36, 37) suggesting that M-CSF may play an important role in the local proliferation and/or functional responses in times of crisis; however, M-CSF message expression induced by cytokines has been found to not always correlate with M-CSF protein secretion.

The human M-CSF gene is localized in the short arm of chromosome 1 at positions p13-p21, and includes 10 exons and 9 introns that span an area of about 21 kb (30, 38, 39). The full-length human M-CSF mRNA encodes a precursor protein of 522 amino acids with a 32 amino acid signal sequence, four potential N-glycosylation sites, a single consensus sequence at Ser-277 for the addition of glycosaminoglycan, 23 amino acids (residues 464-486) encoding for a hydrophobic transmembrane domain, and a sequence of charged amino acids (RWRRR) that appears to function as a stop transfer sequence [(31) and references therein]. In the mouse the M-CSF gene is located in the op region of chromosome 3 in close association with similar genes as its human counterpart (40). The mouse gene encodes for a precursor transcript of 520 amino acids with 60% homology to the human transcript, and displays all of the features outlined above for human M-CSF (31). The highest level of sequence similarity (80.5%) occurs in the N-terminal region (residues 1-149), which has been shown to be essential for biological activity of the M-CSF molecule (41). Significant similarity can also be found in the 5' promoter regions of the human and mouse M-CSF genes. These promoter regions exhibit 80% sequence similarity in the region upstream from the transcription start site emphasizing their importance to gene expression regulatory mechanisms through binding of transcription factors like NF-κB and Sp1 (33, 42, 43). Other than human and mouse, sequences encoding for M-CSF have been identified in rats (44) and bovids (45).

Although M-CSF is encoded by a single gene, several mRNA splice variants ranging in size from 1.5 Kb to 4.4 Kb have been identified [reviewed in (17, 31, 43, 46)]. Alternative splicing of the nuclear RNA precursor can occur in both coding and non-coding regions. Alternative splicing in the 3' non-coding exon 9 and exon 10 regions has implications for the stability of the resulting transcripts. This is directly related to the presence of AU-rich sequences in exon 10 which confer mRNA instability (41, 43, 47-49). Alternative splicing of the coding region in exon 6, in contrast, has implications for the structural characteristics and fate of the resulting M-CSF protein species. mRNA transcripts containing the full-length exon 6 result in protein products that are secreted from the cell in either glycoprotein (~85 kDa – major form detected in human bodily fluids) or proteoglycan forms (16, 50, 51). Both of these secreted forms accumulate in the medium with a doubling time of about 40 min. In contrast, alternative splicing of the 5' end of exon 6 results in the stable expression of a membrane-spanning glycoprotein at the cell surface as the intracellular proteolytic cleavage sites have been spliced out (16). This 68 kDa surface homodimer, however, can be quickly released from the membrane as a 44 kDa species upon phorbol ester stimulation, presumably as a result of proteolytic cleavage at or near the cell surface triggered by protein kinase C activation (52-54).

Analysis of the three-dimensional structure of M-CSF terminal 150 amino acids at 2.5 angstrom resolution indicated that the recombinant human M-CSF dimer contains two bundles of four α-helices laid end-to-end with an interchain disulphide bond (55). The conformation of the monomers is structurally similar to that of other short chain type I cytokines including IL-3 and GM-CSF. Whereas conservation of the amino terminal portion of the growth factor is required for full activity, different species of M-CSF are generated through the alternative splicing mechanisms discussed above, in addition to differential level of glycosylation of the resulting polypeptides. The presence of glycosylation is, however, not required for biological activity of M-CSF. This leads to the formation of biologically active disulfide-linked homodimers ranging in M_r from 44 to >200 kDa, all of which conserve the N-terminal receptor binding domain [reviewed in (17, 46). Overall, the formation of structurally different M-CSF variants appears to lead to differential modes of M-CSF-mediated regulation: whereas the secreted glycoprotein may be involved in humoral regulation of various cellular targets, the membrane-bound surface glycoprotein and the proteoglycan form (by virtue of its chondroitin sulfate chains) appear to be involved in local regulatory mechanisms (16). As such, M-CSF can regulate cell function via direct cell-to-cell contact and/or autocrine, juxtacrine, paracrine, or endocrine mechanisms (17).

3.2.1. The M-CSF receptor

The biological effects of M-CSF are mediated via the high affinity M-CSF receptor (M-CSFR, CD115) (56). M-CSFR, a member of the class III receptor tyrosine kinase family (RTK), is a large integral plasma membrane glycoprotein (M_r 140-150 kDa) with an N-terminal glycosylated extracellular portion containing five repeated immunoglobulin (Ig) domains, a short single transmembrane domain, a juxtamembrane domain, two intracellular kinase domains divided by a unique kinase insert, and a C-terminal domain (57-59). Other members of this family include stem cell growth factor receptor (c-kit) (60), Flt3 (61, 62), platelet-derived growth factor receptor alpha (PDGFα) (63), and PDGFβ (64).

The M-CSF receptor is expressed primarily on cells on the macrophage lineage (56) and, as such, is a useful marker to discriminate between monocytic and granulocytic progenitor cells and their differentiated progeny. It can also be found in osteoclasts (65), placental trophoblasts (66), and mammary epithelial cells during lactogenic differentiation (67). Following injury or in pathological conditions M-CSFR can be found in astrocytes and neurons (68), myeloid leukemic blast cells (69), some breast and ovarian cancers (70), and vascular smooth muscle cells in atheroma (71, 72).

The human *fms* proto-oncogene (c-*fms*) encodes for the M-CSF receptor, a 972 amino acid product very similar to that of the v-fms oncogene, present in the Susan McDonough strain of feline sarcoma virus (SM-FeSV) (73). The human M-CSFR is located on chromosome 5q33.3 (74, 75) 0.5kb downstream from the PDGFR-β polyadenylation signal in tandem in a head-to-tail conformation (76). The M-CSFR gene is 58 kb in length and is composed of 22 exons and 21 introns (77). Expression of the c-*fms* gene is under stringent control by both extracellular and intracellular stimuli, and appears to occur primarily at the level of transcription. Two different tissue-specific promoters differentially dictate the transcription start site of the c-*fms* gene (67, 78, 79). The upstream promoter, located 350 bp from the non-coding exon 1, is expressed in placental trophoblasts and mammary epithelial cells, and is regulated by sex steroid hormones during pregnancy and lactation. The downstream promoter, active in macrophages, their progenitors, and other M-CSFR expressers, mediates the transcription at multiple sites directly upstream from exon 2 (72, 79). In addition, c-*fms* expression can be regulated through mechanisms including transcriptional attenuation, DNA methylation, and regulation by lineage-specific transcription factors such as PU.1 (80-83), Ets1 (84), Ets2 (85), C/EBPα and C/EBPβ (86, 87), AML1 (88, 89), MITF (90), E12 (91), and c-myb (83, 92). Other than in humans, sequences encoding for the M-CSF receptor have been identified from a variety of species including mouse (93, 94), rat (95, 96), cat (97, 98), dog (99, 100), chicken (Burnside, J., Morgan, R.W., and Cogburn, L.A.; unpublished observations), zebrafish (101), puffer fish (102), rainbow trout (Wang, T., Bols, N. and Secombes, C.J.; unpublished observations), and goldfish (Barreda, D.R. and Belosevic, M.; unpublished observations).

3.2.2. Signal transduction and M-CSF receptor

The M-CSF receptor is an integral plasma membrane glycoprotein with an intracellular tyrosine kinase domain through which extracellular signals are transduced and result in downstream signaling cascades. In short, binding of M-CSF to the extracellular ligand-binding receptor domains promotes receptor dimerization and subsequent activation of the intrinsic tyrosine kinase activity that leads to transphosphorylation of specific tyrosine residues in the intracellular portion of the receptor (103, 104). In the human M-CSFR these specific residues are located: a) in the kinase insert (KI) region – Y699, Y708, and Y723; b) in the tyrosine kinase domain closest to the carboxy-terminus – Y809; and c) in the juxtamembrane region – Y561 (58). Equivalent tyrosines are phosphorylated in the murine M-CSFR: Y697, Y706, and Y721 in the KI region; Y807 in the tyrosine kinase domain; and Y559 in the juxtamembrane region (105-107). The tyrosine-phosphorylated receptor then serves as a docking site for a variety of intracellular signaling molecules that contain *src*-homology 2 (SH2) domains. These include, for example, phosphoinositide 3-kinase at Y721, Grb2 at Y697, STAT1 at Y706, and Src at Y559 (based on murine residue numbers). The details of the downstream signaling pathways and the eventual cellular response continue to be under stringent analysis as contrasting responses have been observed depending on the cellular model and experimental approach used [reviewed in (108)]. Following receptor activation and stimulation of downstream signaling cascades the monomeric units of the CSF-1R undergo covalent dimerization via disulphide bonds (109). This is then followed by polyubiquitination of the cytoplasmic domain, kinase inactivation, phosphotyrosine dephosphorylation, internalization via clathrin-coated pits and vesicles, targeting to lysosomes, and destruction of the receptor-ligand complex [reviewed in (31, 103).

3.2.3. Biological activities of M-CSF

The responses mediated by M-CSF binding to its receptor are pleiotropic, generally having implications in the proliferation, differentiation and survival of the responding cell. However, the overall response is dependent on additional factors including cell type, and co-stimulating signals that result from additional extracellular and intracellular events. Although it is now evident that the action of M-CSF is not limited to mononuclear phagocytes, these cells, including osteoclast precursors, remain the main target population (31). In the hematopoietic tissues, for example, this receptor is exclusively found in cells of the macrophage lineage and bone marrow stromal cells readily produce M-CSF, thus nurturing macrophage development in bone marrow specialized niches (56, 110). This selective expression of the M-CSFR also makes it very useful in the identification of macrophages and their hematopoietic progenitors. The level of M-CSFR expression progressively increases from primitive hematopoietic precursors to monocytes and macrophages (31). This is coupled to M-CSF-mediated increase in expression of several differentiation antigens in macrophages (46). As a result M-CSF stimulates the proliferation, differentiation, and survival of multipotential, bipotential, and unipotential hematopoietic progenitors as they progress through the macrophage differentiation pathway. This is supported by analysis of macrophage growth in a variety of *in vitro* models as well as *in vivo*, where administration of M-CSF in mice and non-human primates leads to an increase of up to 10-fold in circulating monocytes as well as macrophages in some areas of the periphery (liver and peritoneal cavity) (111, 112). Conversely, the absence of M-CSF has dire consequences as illustrated by studies of the osteopetrotic (*op/op*) mouse. This mouse lacks active M-CSF production due to a null mutation in coding region of the M-CSF gene, which leads to the generation of a biologically inactive truncated form of the cytokine (113). Due to this mutation, mice have a severe deficiency of osteoclasts and macrophages, absence of teeth, abnormal bone remodeling and osteopetrosis, low body weight, abnormal breast development, decreased fertility, and shortened life-span. Many of these effects can be reversed upon injection of neonatal mice with recombinant M-CSF.

M-CSF is also a key regulator of macrophage function. M-CSF is a potent mediator of monocyte and macrophage activation increasing the chemotactic, phagocytic, and killing macrophage activities (114-120). M-CSF stimulation results in increased production of plasminogen activator (121, 122), prostaglandin E (123), reactive oxygen (124-126) and reactive nitrogen intermediates (127, 128). M-CSF also stimulates the production of several cytokines including G-CSF, GM-CSF, interleukin-1 (IL-1), IL-6, IL-8, TNFα, and interferon (129-136).

M-CSF also plays significant roles outside the hematopoietic system. It has been shown to be important in bone metabolism, atherogenesis, lipoprotein clearance, and in reproduction (17, 30, 31, 46). In the female reproductive tract, for example, M-CSF concentration can be regulated locally, by mechanisms distinct from those modulating its concentration in the circulatory system. In the mouse, M-CSF concentration increases 1000-fold in response to changes in steroid levels during pregnancy (progesterone and estradiol), which induce M-CSF production by the lumenal and glandular secretory cells of the uterus [reviewed in (16)]. In contrast, there is only a 1.4-fold increase in the circulating concentration of M-CSF during the same period. This increase in M-CSF concentration in the female reproductive system appears to mediate blastocyst attachment, trophoblast outgrowth, implantation, and proliferation of the placental tissue (137).

As a result M-CSF has been used as a therapeutic agent in the treatment of various cancers and infectious diseases. Administration of M-CSF to acute myeloid leukemia (AML) patients after consolidation chemotherapies were found to be clinically beneficial as it shortened the periods of neutropenia and thrombopenia following chemotherapy, reduced the incidence and shortened the duration of febrile neutropenia, as well as shortening the period required to finish three courses of intensive consolidation therapy [reviewed in (46)]. Administration of recombinant human (rh) M-CSF also proved effective in the control of invasive fungal infections in patients undergoing bone marrow transplantation, through significant reduction in mortality rates when given in combination with standard antifungal treatments (138). Overall, rhM-CSF treatment alone or in combination with other cytokines effectively increases the numbers and activation of monocytes and macrophages, enhances antibody-dependent cellular cytotoxicity and antibody-independent tumor cell cytolysis, promotes macrophage antimicrobial phagocytic and killing activities, and lowers platelet numbers and cholesterol levels [reviewed in (30)]. It should be noted, however, that elevated levels of M-CSF can also lead to neoplastic transformation (26, 31).

3.2.4. Negative regulation of M-CSF activity

The potent biological activities of cytokines require that effective negative regulatory mechanisms be in place. M-CSF is produced under steady-state conditions and is quickly upregulated in response to a variety of stimuli (30). Activation of the M-CSFR tyrosine kinase activity results in the recruitment and activation of a discrete array of cytoplasmic molecules that initiate specific signaling pathways and mediate an overall cellular response. As discussed above, these events occur quickly in the presence of minute amounts of circulating M-CSF. Consequently, mechanisms have evolved to effectively control the magnitude and duration of this activity, in addition to those already described to regulate gene expression of M-CSF and its receptor.

3.2.4.1. Internalization and degradation of ligand-receptor complexes

M-CSF has been shown to have a short half-life consistent with its critical regulatory role (139). Circulating M-CSF can be effectively cleared by a process of M-CSF receptor-mediated internalization followed by intracellular destruction of the growth factor (139-141). Early experiments determined that liver and splenic macrophages efficiently removed about 94% of the circulating M-CSF, whereas the remaining 6% was filtered through the kidney. Because differentiated macrophages can more efficiently degrade circulating M-CSF this represents an elegant negative-feedback mechanism through which macrophage numbers can be efficiently controlled.

The degradation of internalized M-CSF ligand:receptor complexes is partially mediated through the action of c-Cbl. c-Cbl is a ubiquitin-protein ligase that has been shown to increase the rate of ubiquitination and degradation of several receptor tyrosine kinases, including the receptors for epidermal growth factor, platelet-derived growth factor, and M-CSF (142 and references therein). With respect to the M-CSF receptor, M-CSF stimulation leads to recruitment of c-Cbl to the plasma membrane where it associates via its tyrosine kinase-binding domain with Y973 (mouse) or Y977 (cat) (both equivalent to Y969 in human CSF-1R) in the carboxy-terminus of the autophosphorylated receptor (142-144). Association of c-Cbl with the receptor then leads to ubiquitination, followed by ligand:receptor complex internalization and degradation.

3.2.4.2. Down-regulation of M-CSF receptor signaling

Transphosporylation of specific tyrosine residues in the intracellular domains of the M-CSF receptor results in the generation of docking sites to which a variety of intracellular signaling molecules that contain *src*-homology 2 (SH2) domains can bind. In addition to signaling molecules that positively modulate downstream events, a variety of negative modulators of M-CSF-mediated signaling also associate with the M-CSF receptor. P56^{dok-2}, a member of the Downstream of Tyrosine Kinases (dok) family of docking proteins, for example, is rapidly phosphorylated in response to M-CSF which results in negative regulation of macrophage proliferation (145). Whereas enforced expression of P56^{dok-2} inhibits proliferation of M-NFS-60 myeloid leukemia cells, inhibition of endogenous P56^{dok-2} expression in J774A.1 macrophage-like tumor cells by stable expression of P56^{dok-2} antisense mRNA accelerates cell proliferation (146). Although the mechanisms by which P56^{dok-2} functions are not completely elucidated, it is possible that P56^{dok-2} competes with other substrates of tyrosine kinases thereby preventing downstream signaling pathways. Another regulator of M-CSF activity is the Suppressor of Cytokine Signaling 1 (SOCS1), a member of the SOCS family of molecular adaptors that negatively regulate cytokine signaling pathways (147, 148). SOCS1 directly associates with Y697 and Y721 of the CSF-1R kinase insert domain via its SH2 domain, is tyrosine phosphorylated, and subsequently down-regulates proliferation-inducing signaling cascades presumably through binding and subsequent inhibition of Janus kinase (JAK) family members (149). There are also a number of phosphatases that negatively regulate M-CSF receptor signaling. SHP-1 (Src-homology-2-domain-containing protein tyrosine phosphatase 1) functions to dephosphorylate key intermediates in a number of cytokine and chemokine-mediated receptor signaling pathways (150). SHP-1 appears to regulate M-CSF activity through direct interaction and dephosphorylation of the M-CSF receptor, because macrophages from SHP-1 deficient motheaten (*me/me*) and motheaten viable (*me^{v}/me^{v}*) mice are hyperproliferative in response to M-CSF stimulation and contain hyperphosphorylated M-CSF receptors (151). Two other phosphatases, SHIP (SH2-containing inositol phosphatase) and PTPase (phosphotyrosine protein phosphatase), also appear to reduce the mitogenic response to M-CSF through similar mechanisms (152-156).

In addition to the inhibition of M-CSF-mediated signaling mechanisms responsible for proliferation and survival, two proteins, Mona and FMIP, have been shown to regulate M-CSF-mediated differentiation pathways. Mona, for monocytic adaptor, is a novel adaptor protein (M_r 38 kDa) containing one SH2 and two SH3 domains (157). Mona over-expression in murine bone marrow cells strongly decreased their differentiation to macrophages in response to M-CSF stimulation (157). The mechanism appears to involve inhibition of Grb2 (growth factor receptor bound 2) by competing for the phosphorylated Y697 SH2 binding site, followed by the association with the DOS/Gab family member Gab3, and the downstream inhibition of the Ras pathway (158). FMIP, a novel FMS-interacting 78 kDa protein, has also recently been identified to specifically bind to the cytoplasmic domain of activated M-CSF receptors (159). Over-expression of FMIP in a bipotential myeloid progenitor cell line, FDC-P1Mac11, prevented M-CSF-induced macrophage differentiation; instead these cells differentiated into granulocytes thus suggesting a possible critical role in the regulation of commitment and differentiation of macrophage/granulocyte precursors.

3.2.4.3. Synthesis and release of soluble M-CSF receptors

An additional mechanism for the regulation of soluble growth factors is through inhibition of the binding to their corresponding receptors on the target cell. This could be achieved through the production of a competitive inhibitor, like that for IL-1, which has been described to bind to the IL-1 receptor but not to activate downstream signaling events (160-165). To our knowledge, no competitive inhibitors for M-CSF or the other colony-stimulating factors have been identified. A second mechanism to prevent binding of soluble growth factors to their corresponding ligand is through the production and secretion of soluble receptors that will bind the ligand thus preventing its association with the cellular-bound receptors. Soluble forms for several cytokine receptors have been identified, including those for IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, G-CSF, GM-CSF, type I and II IFNs, TNF, GH, Epo, and LIF (166-173). These soluble receptors can result from alternative splicing of the mRNA species, which leads to the production of a truncated protein that has the extracellular ligand binding regions of the receptor, but lacks the cellular membrane anchoring domains. Examples of this include IL-4Rα, IL-5Rα, IL-

6Rα, IL-7Rα, IFNαR β chain, and the GM-CSFRα. Alternatively, the full-length membrane-associated receptor can be proteolytically cleaved and released from the cell surface as has been shown for IL-2Rα, GM-CSFRα, TNFRI, and TNFRII. No cases have been identified whereby the soluble receptor is encoded by a distinct gene from the membrane-associated form of the receptor.

As discussed above the mechanisms identified to control M-CSF activity include the active internalization and degradation of ligand:receptor complexes by differentiated macrophages, the regulation of signal transduction pathways downstream of the M-CSF receptor, as well as the induction of negative feedback mechanisms involving the release of M-CSF production inhibitory factors like IFN-β (10) and TNF-α (174, 175) upon M-CSF stimulation. Recent studies in our laboratory also point to an additional mechanism of M-CSF down-modulation: through the production of soluble M-CSFR. Using a differential macro array analysis of goldfish macrophage genes expressed during differentiation *in vitro*, we identified a transcript encoding for a short form of the M-CSFR. This transcript contains a start codon, a predicted 16 amino acid signal peptide, exons 2-4 of the M-CSF gene, a stop codon, polyadenylation signals, and a poly-A tail (Barreda and Belosevic, unpublished observations). In accordance with the corresponding region in human and mouse M-CSFR our transcript encodes for three putative N-glycosylation sites, but only two of the five N-terminal immunoglobulin-like (Ig-like) domains. Interestingly, it is only the three N-terminal disulphide bond-linked immunoglobulin-like domains that have been shown to mediate M-CSF binding. Wang and colleagues (54) constructed a series of truncated receptors containing different portions of the murine M-CSFR. Characterization of the ligand-binding ability of each of these constructs determined that the extracellular portion of the M-CSFR functions independently of the transmembrane and intracellular portions in M-CSF binding. M-CSF binding affinity progressively increased as the first, second, and third N-terminal Ig-like domains were included, and was greatest when all three Ig-like domains were present in the recombinant protein. In contrast, the binding affinity for M-CSF decreased significantly (almost half) when the remaining fourth and fifth immunoglobulin-like domains were included, confirming that these domains may be more important to conformational changes that mediate downstream signaling events than M-CSF binding.

Other receptors, including the PDGF receptor (176), fibroblast growth factor receptor (177), as well as the extracellular matrix receptor ICAM-1 (178, 179), also display similar preferential binding of their respective ligands to the first three amino-terminal domains.

Interestingly, the transcript encoding for our predicted soluble form of the M-CSFR was found in a *senescence phase* goldfish macrophage cDNA library. Previous characterization of the development of primary macrophages from goldfish hematopoietic tissues in response to endogenous growth factors indicated that following a period of active proliferation and differentiation events (the *proliferative phase*), macrophage cultures then entered a *senescence phase* characterized by cessation of proliferation and differentiation events, a shift in flow cytometric parameters, cellular clumping, debris formation, and cellular death through a process of apoptosis (180). Moreover, cell-conditioned medium collected from *senescence phase* primary kidney macrophage (PKM) cultures inhibited the growth of subsequent PKM cultures. Our working hypothesis is that this putative soluble form of the M-CSF receptor may play a central role in the clearance of the M-CSF ligand from the culture supernatants leading to the cessation of cellular growth and apoptotic death commonly observed in the *senescence phase* of PKM development. We are currently testing this hypothesis through the expression of the soluble M-CSFR transcript and the characterization of the effects that the recombinant protein has on macrophage growth, the rate of entrance into the *senescence phase*, as well as its ability to bind soluble macrophage growth factors.

3.3. Granulocyte colony-stimulating factor (G-CSF)

Neutrophils, the major subset of the polymorphonuclear phagocytes, represent 50-75% of the total circulating leukocytes, and play a central role in inflammation and immune responses to invading bacteria, fungi, protozoa, viruses, and tumor cells (181). They have a short half-life (4-10 h in circulation followed by 1-2 days in tissues) and, therefore, need to be constantly replenished from a pluripotent pool of undifferentiated hematopoietic progenitors in the bone marrow. The granulocyte colony-stimulating factor (G-CSF) is, therefore, central to neutrophil-based immune defenses through its regulatory role in the growth, differentiation, survival, and activation of neutrophils and their precursors (29). G-CSF also stimulates the growth of early hematopoietic progenitors in combination with IL-3 (synergy), and mediates the proliferation and migration of endothelial cells.

G-CSF was first purified by D. Metcalf and his colleagues from mouse lung-conditioned medium as a 25kDa glycoprotein with a pI=4.5-5.8; about 5 µg were obtained from 800 mice (182). The purified factor was shown to stimulate proliferation of normal granulocytic progenitor cells, and induce the terminal differentiation of murine myelomonocytic leukemia WEHI-3BD$^+$ cells. Subsequently, human G-CSF was purified from a squamous cell carcinoma (CHU-2 line) (183), a T-leukemia cell line (Mo), and a bladder carcinoma cell line (5637) (184), in all cases yielding a hydrophobic acidic glycoprotein of 19-22 kDa and a pI=5.5-6.1 depending on the degree of sialylation (127). In normal serum G-CSF is detected in low concentrations of 25 ± 20 pg/ml (185).

The human G-CSF gene is located on chromosome 17 at positions q21-22, and contains five exons and four introns that span an area of approximately 2.5 kb (186-189). This is in contrast to several other growth factors whose genes are located in the long arm of chromosome 5: IL-3 (5q23-q32), IL-4 (5q23-q32), IL-5 (5q31-q33), FGF (5q31-q33), GM-CSF (5q23-q31), M-CSF receptor (5q23-q34), PDGF-R (5q21-q32) [reviewed in (127)]. The full-length human G-CSF mRNA encodes a precursor protein of 204 amino acids, with a 30 amino acid signal peptide, two predicted interchain disulphide bridges, and no sites for N-asparagine-linked glycosylation. Cleavage of the signal peptide yields a mature 174 amino acid G-CSF protein with slightly higher than the predicted molecular weight (18.7 kDa) presumably as a result of O-linked glycosylation at Thr-133 (188, 190). The sugar moiety, however, is not required for biological activity as the recombinant G-CSF protein produced in *E. coli* is as active as the native glycosylated protein (191). The role for the hydrophilic O-linked sugar chain appears to be the prevention of G-CSF aggregation by increasing the solubility and stability of the highly hydrophobic G-CSF molecule at neutral pH (192). Disulphide bond formation between Cys36-Cys42 and Cys64-Cys74, on the other hand, is required for biological activity as this allows for structural stability and proper folding of this long chain type I cytokine (173, 193). Analysis of the G-CSF gene in the mouse indicated a similar exon-intron structure, as well as a mature 178 amino acid protein displaying 73.6% identity to its human counterpart (189, 194-196). Not surprisingly, there is significant cross reactivity between human and mouse G-CSFs (197-199). Recombinant human G-CSF, for example, induces the terminal differentiation of murine WEHI 3B (D+) cells, the proliferation of murine NFS-60 cells, and colony formation from murine bone marrow cells (191, 200). Moreover, subcutaneous administration of rhG-CSF into mice strongly stimulated granulopoiesis and splenomegaly (200). Conversely, recombinant mouse G-CSF or G-CSF from a murine marrow stromal cell line MS-5 synergized with murine stem cell factor (SCF) to stimulate the growth of Lin$^-$CD34$^+$CD38$^-$ human cord blood cells (201).

The human G-CSF gene but not the mouse G-CSF gene produces an alternatively spliced mRNA transcript (189). This alternatively spliced form encodes for a 177 amino acid product that results from alternative use of a 5' splice donor sequence in the second intron of the G-CSF gene. Insertion of this Val-Ser-Glu tripeptide between human G-CSF residues 35 and 36 results in a protein product with about 10-fold less specific activity than the 174 amino acid species, but constitutes only a small population of the human G-CSF mRNA population (188, 189). Because this insertion results in structural modifications around disulphide bond-linked Cys-36 it is possible that this results in the lower specific activity displayed by this G-CSF form. This is supported by the identification of neutralizing monoclonal antibodies that recognize, among others, a disulphide-linked loop formed through Cys-36-Cys42 bonding (202). Overall, the characterization of the epitopes recognized by these antibodies (both neutralizing and non-neutralizing) as well as site-directed mutagenesis studies implicate residues 20-46 and the carboxy-terminus of G-CSF in receptor binding (202, 203).

Regulation of G-CSF gene expression is achieved at the level of transcription as well as post-transcriptionally. The transcriptional activation of the G-CSF gene is mediated through a well-conserved 300 nucleotide region located upstream from the G-CSF ATG start codon (5, 191). Three regulatory elements have been characterized within this region: G-CSF promoter element-1 (GPE1), GPE2, and GPE3. GPE1 encompasses an NF-κB-like CSF box and a NF-IL-6 binding site. GPE2 contains the octamer transcription factor binding site. GPE3 encompasses a unique 19 nucleotide sequence element beginning at position –98. All three G-CSF promoter elements are required for constitutive gene expression in the tumor cell line CHU-2 as well as LPS inducible gene expression in macrophages. On the other hand, IL-1 and TNF-α-mediated regulation of G-CSF gene transcriptional activity is dependent on the binding domains within the GPE1 and GPE2 regions, and does not appear to require GPE3 nor two additional sequences: a downstream CK2 element at position –69, and an upstream PU.1 binding site at position –235. Post-transcriptional regulation of G-CSF expression is mediated by the presence of AU-rich elements (AREs). Like several other cytokine mRNAs, G-CSF mRNA contains AU-rich sequences in the 3' non-coding region that confer transcript instability (49). Stimulation with agents such as bacterial LPS, IL-1, and TNF-α, however, confers transient mRNA stability, presumably through interference with RNA-binding proteins or RNase activity (204-206).

In terms of the evolutionary conservation of G-CSF, sequence information about the G-CSF gene and protein is available for a variety of species. Following the identification of G-CSF in humans and mice, porcine (207), rat (208), bovine (209, 210), feline (211, 212), ovine (213), and equine (Noronha, L.E., *et al*; unpublished observations) G-CSF have been characterized and share a high degree of amino acid sequence similarity (65-90%). In addition, human G-CSF shows significant similarity (56% identity) with the chicken myelomonocytic growth factor (cMGF) (214, 215). cMGF is a secreted chicken glycoprotein, originally isolated from a transformed macrophage cell line, which induces the proliferation and differentiation of granulocytes and macrophages from transformed myeloblasts and normal chick bone marrow at picomolar concentrations. Characterization of cMGF revealed that it is a remarkably stable glycoprotein ranging in size from 23-200 kDa that also shares significant similarity with human interleukin 6 (IL-6; 39% homology). It remains unclear whether cMGF represents an evolutionary precursor to both mammalian G-CSF and IL-6, but the degree of sequence similarity, predicted tertiary structure, and characterization of its receptor do support the possibility of a common origin.

G-CSF is generated by a variety of cells, of which monocytes and macrophages are considered to be the major producers (216). Monocyte stimulation with bacterial lipopolyssacharide (LPS), phorbol myristate acetate (PMA), or cyclohexamide does not result in an increase in the transcription rate of the G-CSF gene, but results in the stabilization of G-CSF mRNA (217-221). Increased production and release of G-CSF has been observed following stimulation with LPS (221-224), PHA and PMA (225), IL-1 (222), IL-3 (226), IL-4 (227), GM-CSF (222, 226), M-CSF (228, 229), TNF-α and IFN-γ (230), and *Mycobacterium avium* (231). In addition, stimulation with LPS or a variety of cytokines including TNF-α, IFN-γ, IL-1, and GM-CSF have been shown to promote G-CSF production by fibroblasts, endothelial cells, T-lymphocytes, and polymorphonuclear granulocytes (232-237).

3.3.1. The G-CSF receptor

All of the biological effects of G-CSF are mediated via the G-CSF receptor (G-CSFR), a member of the type I cytokine receptor superfamily. This receptor, like that for M-CSF, binds its ligand with high affinity as a homodimer. Unlike the M-CSF receptor, however, the G-CSFR does not have intrinsic tyrosine kinase activity and therefore relies on cytoplasmic enzymes for the stimulation of downstream signaling events.

The G-CSF receptor is primarily expressed in neutrophilic progenitors and mature neutrophils, and its main function is to transmit signals for proliferation, differentiation, and survival of these cells (238). It can also be found on monocytes, mature platelets, and various non-hematopoietic cells and tissues, including endothelial cells, placenta, trophoblastic cells, and several myeloid leukemia cells (NFS-60, WEHI-3BD$^+$, HL-60) (239, 240). Maturation of neutrophil precursors leads to an increase in G-CSFR expression, with mature human neutrophils displaying approximately 200-1000 receptors per cell (241-243). Consistent with these reports G-CSFR expression can be up-regulated following treatment of immature myeloid cells with G-CSF, as well as treatment of acute promyelocytic leukemia cell lines with all-*trans* retinoic acid, which has been shown to be a strong inducer of neutrophilic differentiation (244, 245). In contrast, several agents shown to activate neutrophils including GM-CSF, TNF, LPS, C5a, f-met-leu-phe, and phorbol esters (TPA) have been shown to down-regulate G-CSF receptor expression (246, 247).

The human G-CSF receptor (CD114) is encoded by a single gene located on the short arm of chromosome 1 at positions p35-p34.3, and includes 17 exons of relatively short length that span an area of about 16.5 kb (248, 249). Exons 3-17 encode for the mature G-CSFR protein. In the mouse the G-CSFR-encoding functional gene (*Csfgr*) is localized to the distal region of murine chromosome 4 (D2.2) and displays similar exon-intron organization than its human counterpart despite having a significantly larger intron 14 (250). In contrast to the human G-CSFR and other cytokine receptors, the mouse genome possesses two genes encoding for the G-CSFR: one encoding for the functional receptor and a second processed pseudogene which lacks all the introns, exons 1-3, and exons 7-8 of the functional gene. Molecular analysis of the cDNAs encoding the murine and human G-CSFRs has shown that the murine and human G-CSFR proteins are single transmembrane polypeptides of 812 and 813 amino acids, respectively, with an overall homology of 72.5% at the nucleotide level and 62.5% at the amino acid level (251). The most closely related member of the type I cytokine receptor family is gp130, the common signal transducing subunit of IL-6, LIF, and other cytokine receptors (252). Other than human and mouse, sequences encoding for the G-CSFR have been identified in bovine (253), chicken (Morgan, R.W., Burnside, J.; unpublished observations), winter flounder (Douglas, S.E., *et al*; unpublished observations), and Xenopus (Strausberg, R; unpublished observations).

Post-transcriptional modification of G-CSFR leads to the formation of five different mRNA species by alternative splicing. The predominant full-length form (class I) leads to the production of a single chain type I transmembrane molecule containing 604 amino acids in the extracellular region, 26 amino acids in the transmembrane region, and 183 amino acids in the intracellular region (239). The extracellular portion of the receptor is composed of six structural domains extensively glycosylated at nine potential N-glycosylation sites, and contains 19 conserved cysteine residues that appear to mediate the formation of 8 disulphide bonds. From the amino terminus, there is an immunoglobulin-like (Ig-like) domain, a cytokine receptor homology (CRH) module with four conserved cysteine residues and a WSXWS motif that are essential for ligand binding, and three fibronectin type III (FN III) domains (254-256). The transmembrane domain is followed by a cytoplasmic domain that contains two loosely conserved regions of sequence homology with other cytokine receptors referred to as Box1 and Box2; this region appears to be essential for signal transduction of mitogenic/proliferation signals. In addition, a membrane distal domain, Box3, contains a STAT3 binding site and appears to be essential for signal transduction of cellular differentiation signals (257-259). Based on its amino acid sequence the predicted M_r for the polypeptide is 90 kDa; however, the native protein expresses a M_r of 130-150 kDa as a result of differential glycosylation on its extracellular domain (243, 260, 261). The remaining forms of the G-CSFR, generated through alternative splicing (class II-V) all have identical extracellular domains but differ in their C-terminal sequences (238, 251). The class II encodes for a soluble form of the G-CSFR generated by a frameshift mutation that results in deletion of the transmembrane domain. Although the physiological relevance of this soluble G-CSFR is unknown, it appears that it may function as a negative modulator of G-CSF through competition with the membrane bound receptors (refer to the "Negative modulation of G-CSF" section below). The class III receptor contains a 27 amino acid insertion in the region between Box1 and Box2. The class IV receptor contains a deletion of the 87 carboxy-terminal amino acids of the full-length receptor, and contains a distinct 34 amino acid carboxy-terminal tail. The class V receptor contains a larger deletion directly downstream of Box2, but contains an identical 34 amino acid carboxy terminus to class IV (235, 248).

As described above the G-CSFR is differentially expressed in a differentiation stage and tissue-specific manner. Analysis of the human G-CSFR indicates that transcriptional activation can be effectively modulated by a number of transcription factors. A binding site for the CCAAT/enhancer binding protein-α (C/EBPα) at bp –49, and two recognition sequences for PU.1 (spi-1) between +36 and +43 were identified to be important for G-CSFR promoter activation (262). Mutation of sequences within either of these sites resulted in significant reduction of G-CSFR promoter activity. Multiple putative binding sites for transcription factors AP-1, AP-2, and GF-1 have also been identified, but not yet characterized (249). In addition, an 18 nucleotide element located about 110 bp upstream from the major transcription initiation site is homologous to sequences found in the promoters for two neutrophil-specific enzyme genes, myeloperoxidase and neutrophil elastase, suggesting that a common trans-regulatory factor may regulate transcriptional activation of the promoter in all three genes.

3.3.2. Signal transduction and G-CSF receptor

The generation of signal transduction cascades and subsequent G-CSF-mediated biological functions requires specific interactions between the incoming G-CSF protein and the cell surface G-CSFR. Structural and mutagenesis experiments indicate that G-CSF binding occurs via the CRH module and a ligand-binding site on the Ig-like domain at the N-terminus of the protein (263, 264). The three FN III repeats between the CRH region and the membrane surface are not directly involved in ligand binding but may play an important role in receptor dimer stabilization (255). Binding of G-CSF to its receptor promotes homodimerization of receptor chains resulting in the formation of a tetrameric complex containing two ligand and two receptor molecules (238, 265). Although low affinity binding can occur between G-CSF and monomeric G-CSFR proteins (Kd 2.6-4.2 nM), high affinity binding (Kd 120-360 pM) mediated by formation of the tetrameric complex appears to be necessary for signal transduction mediated by the G-CSFR.

Oligomerization on the receptor chains leads to rapid phosphorylation of four tyrosines in the cytoplasmic region of the human G-CSFR, Y704, Y729, Y744, and Y764; similar tyrosine residues have been identified in the mouse G-CSFR (255, 260). These phosphorylated tyrosine residues then serve as docking sites for signaling molecules containing SH2 or phosphotyrosine binding domains (266-268). Overall, multiple signals emanate from the receptor tyrosine residues that control proliferation, differentiation, and survival (269). In addition, signals independent of the receptor tyrosines, modulated through a membrane-distal cytoplasmic domain in the G-CSFR, also contribute to differentiation and survival. Important signaling molecules that mediate transduction of G-CSFR responses include the Janus tyrosine kinases JAK1, JAK2, an Tyk2, the Src kinases $p55^{lyn}$ and $p56/59^{hck}$, the signal transducer and activator of transcription (STAT) proteins STAT1, STAT3, and STAT5, as well as components of the $p21^{ras}$/Raf/mitogen-activated protein kinase pathway [reviewed in (238, 251, 269, 270)].

3.3.3. Biological activities of G-CSF and its receptor

The granulocyte colony-stimulating factor is the primary cytokine controlling the survival, proliferation, and differentiation of cells along the granulocytic pathway (191, 199, 271). In fact, although both IL-3 and GM-CSF can support the growth of neutrophil progenitors in the early stages of development, G-CSF is necessary for their terminal differentiation to mature neutrophilic granulocytes. The essential role of G-CSF in neutrophil hematopoiesis is clearly shown *in vivo*. For example, dogs injected with human G-CSF developed antibodies that cross-neutralized canine G-CSF and resulted in the development of neutropenia (272). Moreover, injection of the antibody-containing serum into other dogs also resulted in comparable neutropenia. Similarly, mice containing homozygous inactivation of the G-CSF gene, thereby inhibiting normal G-CSF production, only contained 20-30% of the normal circulating neutrophil numbers, and showed a 50% reduction in the number of granulocyte-macrophage progenitor cells, as well as immature and mature granulocytes in the bone marrow (273).

The role of G-CSF in up-regulation of myelopoiesis, however, is most apparent under stress conditions. Endotoxin and inflammatory mediators strongly stimulate transcription levels of the G-CSF gene as well as protein production (274). This results in dramatic increases in circulating G-CSF concentration, and up to 10-fold increases in neutrophil numbers, while other blood lineages remain largely unaffected (275). The larger neutrophil presence is the result of an increase in the rate of neutrophil production in the bone marrow resulting, in part, from a shortened maturation time, stimulation of neutrophil and progenitor release from the bone marrow and augmented migration into the tissues, as well as the enhancement of neutrophil survival through promotion of anti-apoptotic mechanisms. G-CSF$^{-/-}$ mice, in contrast, are significantly impaired in their ability to control infections, such as with *Listeria monocytogenes*, and are unable to show marked increases in peripheral blood neutrophil numbers (273).

In addition to stimulation of proliferation and differentiation in neutrophilic granulocyte precursors and their progeny, G-CSF has also been shown to have a profound influence in the biological functions of mature neutrophils (127). G-CSF stimulates neutrophil phagocytic and chemotactic activity, increases the production of reactive oxygen intermediates in response to bacterial peptides, and enhances antibody-mediated cellular cytotoxicity (ADCC) against antibody-coated tumor target cells (276). G-CSF also stimulates the release of arachidonic acid, the production of leukocyte alkaline phosphatase and myeloperoxidase, and appears to play a role in the process of nuclear segmentation in maturing neutrophils (277-279).

As with other cytokines, the effects of G-CSF are pluripotent and modulate the responses of cellular types outside the neutrophil lineage. As mentioned above G-CSF plays an important role in mobilizing hemopoietic stem cells from bone marrow to circulation *in vivo*. In contrast to its stimulatory effects on neutrophils, G-CSF predominantly down-regulates inflammatory responses by reducing proinflammatory cytokine formation in activated monocytes and macrophages (216, 280). G-CSF stimulation promotes the proliferation and migration of endothelial cells *in vitro*, and augments the secondary aggregation of platelets induced by a low concentration of adenosine diphosphate (281, 282). In addition, G-CSF treatment increases peripheral lymphocyte numbers and has an indirect modulatory effect on lymphokine release and adaptive immune responses (280).

The wide-array of G-CSF-mediated effects in the modulation of granulocyte hematopoiesis and in the control of blood cell responses has led to its use as a therapeutic agent. Recombinant G-CSF has been used clinically to accelerate hematopoietic recovery following transplantation, in part to reduce the risk of severe infections or hemorrhage (275). G-CSF treatment reduces the duration of neutropenia and enhances hematopoietic reconstitution, and has therefore been found useful in the recovery from chemotherapy and bone marrow transplantation (283-285). Part of the attraction to G-CSF-related treatments is the comparably few negative side effects that can be identified, even when excess levels are present *in vivo*. Mice with excess G-CSF, for example, did not exhibit a reduced life-span or marked disease states such as leukemic transformation (286). In fact, detectable changes were limited to marked hyperplasia of granulocyte populations and an increased number of mature granulocytes in organs such as the lung and kidney. Overall, since its introduction about 17 years ago, G-CSF has become one of the most successful recombinant proteins in clinical use, with more than 1.4 million patients having been treated (280).

3.3.4. Negative regulation of G-CSF activity

The activation of signaling cascades by G-CSF, similar to other cytokines, is transient as a result of several cellular mechanisms that tightly regulate them. The mean elimination half-life of circulating G-CSF is about 163 minutes in normal donors, and results in low G-CSF concentrations of about 25 ± 20 pg/ml in normal serum (5, 185).

3.3.4.1. Internalization and degradation of ligand-receptor complexes

Circulating G-CSF is actively cleared by granulocytes through a process of ligand-receptor internalization and degradation (287-290). This is mediated by immature myeloid cells and mature neutrophils, but is more effective at later stages of differentiation where the number of receptors per cell is greater. As such, it provides an effective negative feedback mechanism aimed at controlling myelopoiesis and maintaining homeostasis. In humans, for example, there is an inverse relationship between the serum G-CSF levels and the number of circulating neutrophils (288). In contrast, inhibition of this control mechanism has serious consequences for the modulation of G-CSF-mediated responses. Deletion of a critical internalization domain in the G-CSFR leads to impaired ligand internalization, defective receptor down-modulation, prolonged receptor activation, and enhanced growth signaling at the expense of cellular maturation (291, 292). The presence of such truncated G-CSFRs have been identified in a subset of severe congenial neutropenia (SCN) patients, where nonsense mutations introduce a premature stop codon that leads to deletion of the distal cytoplasmic tail of the G-CSFR (293-295). Specifically, it appears that the loss of a di-leucine motif in Box3 of the G-CSFR cytoplasmic domain is partially responsible for defective receptor internalization following ligand binding (292).

The mechanisms mediating G-CSF/G-CSFR complex internalization and degradation also have practical implications for the use of recombinant G-CSF as a therapeutic agent. For example, during the treatment of cancer patients following chemotherapy rhG-CSF increases both the number of circulating neutrophils as well as the number of receptors per cell, thus effectively modulating its own clearance (296).

3.3.4.2. Down-regulation of G-CSF receptor signaling

The SH2-containing inositol phosphatase (SHIP) effectively down-regulates the G-CSF mitogenic signal (297). After treatment with G-CSF, phosphorylation of Tyr764 leads to Shc binding to the distal end of the cytoplasmic G-CSFR domain and recruitment of SHIP. Activation of SHIP appears to induce PI3-kinase substrate degradation and results in down-regulation of the proliferation stimulus. The increased proliferation of G-CSFR Y764→F764 mutants further supports this mechanism, presumably through their inability to recruit Shc/SHIP. The protein tyrosine phosphatase SHP-1 is another candidate for negative regulation of G-CSF receptor signaling. SHIP-1 protein levels are up-regulated in response to G-CSF stimulation and appear to promote differentiation of murine 32DC13 granulocytic precursor cells through inhibition of proliferation signals (298). Whereas enforced expression of SHP-1 in these cells lead to a decrease in proliferation and increased granulocytic differentiation, the introduction of a catalytically inactive mutant resulted in increased proliferation and a delay in cellular differentiation (295).

Another mechanism responsible for down-regulation of G-CSFR signaling involves members of the suppressor of cytokine signaling (SOCS) family. SOCS-3 expression is induced in response to G-CSF stimulation of polymorphonuclear neutrophils and the myeloid precursor cell line U937, and inhibits G-CSFR-mediated signaling (299). Specifically, SOCS-3 is recruited to the G-CSFR following G-CSF binding and specifically interacts with the phosphorylated Y729 subsequently inhibiting STAT activation. The expression of another member of the SOCS family, SOCS-1, is also up-regulated in myeloid precursor cells, polymorphonuclear phagocytes, and the murine myeloid cell line NFS-60 in response to G-CSF stimulation (299, 300). SOCS-1 effectively inhibits STAT-mediated induction of gene expression following G-CSF stimulation, in a reporter gene assay (299). Finally, c-Cbl has been implicated in the modulation of JAK/STAT signaling following G-CSF stimulation, but the mechanisms through which it may regulate cellular growth remain unclear (301).

3.3.4.3. Synthesis and release of soluble G-CSF receptors

In the section outlining the mechanisms of M-CSF down-regulation we described the possible modulation of M-CSF activity via a soluble M-CSF receptor, as has been described for several other cytokines. Similarly, a cDNA was isolated from myeloid precursor U937 cells that encoded for a truncated soluble form of the G-CSF receptor (sG-CSFR) (302). Immunoblot analysis using antibodies against the amino-terminal region of the human G-CSFR extracellular domain subsequently indicated that two isoforms of the human G-CSFR could be identified in human serum that bind recombinant human G-CSF (170). Interestingly, in contrast to full-length G-CSFR that was expressed in neutrophils during maturation (blasts, promyelocytes, myelocytes, metamyelocytes, and mature neutrophils), the mRNA encoding for the sG-CSFR was only detectable at later stages of neutrophil differentiation. Although the exact role of the sG-CSFR in hematopoiesis regulation remains unclear, one possibility is that its production by mature cells may be part of a negative feedback mechanism geared to the control of myelopoiesis and the maintenance of homeostasis. This is supported by characterization of the biological activity of a recombinant truncated form of the murine G-CSFR containing only the extracellular domain (303). This soluble G-CSFR inhibited G-CSF induction of normal bone marrow colony formation and leukemic progenitor cell proliferation from patients with acute myeloblastic leukemia. Another possibility is that the sG-CSFR may act as a positive, rather than negative, modulator of G-CSF-mediated responses through the stabilization and/or prevention of ligand degradation as has been shown for soluble growth hormone receptors (169, 304, 305). For example, Ku and colleagues (306) reported that a recombinant form containing the extracellular domain of the G-CSFR synergized with steel factor or with the Flt3/Flk2 ligand to promote the proliferation of primitive hematopoietic progenitors.

3.4. Granulocyte macrophage colony-stimulating factor (GM-CSF)

During the early studies on colony-stimulating activity, lung tissue became of special interest because it contained high levels of a CSF activity that stimulated the formation of both granulocyte and macrophage colonies in semisolid medium (307). In 1977, GM-CSF was purified from lung tissues of mice that had been pre-injected with endotoxin; however, due to its strong biological activity at minute amounts, starting material from 100,000 mice was required (308, 309). Human GM-CSF was subsequently purified from medium conditioned by a Mo human T-lymphoblast cell line using similar multiple sequential fractionation procedures (310). Both human and murine GM-CSFs were identified as glycoproteins that displayed similar molecular weight (24-33 kDa).

The human GM-CSF gene is located in the long-arm of chromosome 5 at positions q23-q31, and contains four exons and 3 introns that span an area of approximately 2.5 kb (311). It is positioned in tandem to the gene for interleukin 3 (IL-3) and in close proximity to the genes for IL-4, IL-5, IL-9, and the M-CSFR (312). In the mouse the GM-CSF gene is located on chromosome 11, at bands A5 to B1 (196, 313-315). The full-length human GM-CSF mRNA encodes a precursor protein of 144 amino acids, with a 17 amino acid hydrophobic leader sequence, two predicted interchain disulphide bridges (Cys 54-96 and Cys88-121), and two potential N-glycosylation sites at Asn27 and Asn37 that yield a core protein of 14.7 kDa [reviewed in (5)]. Cleavage of the signal peptide yields a mature 127 amino acid GM-CSF protein that is expressed as protein species ranging in molecular weight from 14.7 to 33 kDa presumably due to complex acidic type N-glycosylation although additional sites of O-glycosylation have also been described at the N-terminus (Ser9 and Thr10) (316, 317). The full-length murine GM-CSF mRNA encodes for a similar molecule containing a 17 amino acid hydrophobic leader sequence that, upon cleavage, results in the production of a 124 amino acid protein with a core polypeptide molecular weight of 14.4 kDa, two predicted intramolecular disulphide bonds (Cys 51-93 and Cys 85-118), and two potential N-glycosylation sites at Asn 66 and Asn75. Non-glycosylated forms of protein have the full range of biological activities characteristic of the glycosylated forms; surprisingly, the specific biological activity of non-glycosylated forms is over 10-fold greater than that of glycosylated GM-CSF (317-319). The intramolecular disulphide bonds contribute to the stability of the tertiary structure, but only the disulphide bond between Cys54-96 is required for biological activity (320, 321). Human and murine GM-CSF proteins only share 52% amino acid identity and do not cross-react in their biological activities. Other than human and mouse, sequences encoding for GM-CSF from a variety of species have been identified including gibbon (322), macaque (323), bovine (324), ovine (325, 326), porcine (327), canine (328), cat (Hughes, K.J. and O'Reilly, K.L.; unpublished observations), rat (329), gerbil (330) and woodchuck (331). Although significant homology can be detected in both nucleotide and protein sequences, species cross-reactivity is limited.

Analysis of the three-dimensional structure of non-glycosylated GM-CSF at 2.4 angstrom resolution indicated that GM-CSF displays a two-stranded antiparallel β-sheet with an open bundle of four α-helices similar to M-CSF, G-CSF, IL-2, IL-4, IFNβ and growth hormone placing it in the short-chain α-helical bundle type I cytokine family (332, 333). The structural elements of GM-CSF responsible for binding to its receptor and necessary for biological activity, have been characterized by several techniques including scanning mutagenesis, generation of interspecies chimeras, mapping of epitopes by using neutralizing monoclonal antibodies, and peptide synthesis [reviewed in (5, 334)]. In short, whereas helix B does not appear to be essential for biological activity, residues 21-31 (in helix A) and 78-94 (in helix C) are critical for high-affinity receptor binding and biological activity. Although helix D does appear to contribute to receptor binding and biological activity its contribution appear less that that of helices A and C. Incoming GM-CSF binds to specific residues on the GM-CSFR including Arg-280 in the membrane proximal region of the GM-CSFR α chain. Glu-21 of helix A has also been implicated in high affinity binding to the β-chain of the GM-CSF receptor, presumably at His-367 (335-337).

Under normal conditions GM-CSF can be detected in serum at concentrations ranging from 20-100 pg/ml. GM-CSF is produced by a variety of cell types including macrophages (338, 339), T lymphocytes (340-342), fibroblasts (235, 343, 344), endothelial cells (232, 234, 345-347), stromal cells (348-350), keratinocytes (351), placental cells (352, 353), astrocytes (354), B-lymphocytes (355), mesothelial cells (356), osteoblasts (357), mast cells (358, 359), eosinophils (360), neutrophils (360), and blast cells (361). In some cases, production of GM-CSF is constitutive as in a number of tumor cells lines; however, in most cases it requires stimulation of the producing cell with other cytokines, antigens or inflammatory agents (5, 334).

Regulation of GM-CSF gene expression is achieved at the level of transcription as well as post-transcriptionally. At the transcriptional level GM-CSF gene expression is modulated by multiple promoter and enhancer elements (5). Several of these can be found upstream of the TATA box including a GC-rich region (GC box), an NF-κB binding site, and two conserved cytokine consensus elements, CK-1 and CK-2 (also referred to as conserved lymphokine elements CLE-1 and CLE-2). The GC box appears to be responsible for constitutive GM-CSF expression, whereas CK-1 and CK-2 elements appear to bind to inducible factors (362, 363). There is also a sequence element homologous to sequences in IL-4, IL-5, and G-CSF genes, the CATTA/T box or CLE0 box, that appears to bind transcription factors including NF-AT and AP-1 (364, 365). Post-transcriptional regulation of GM-CSF expression is mediated by the presence of AU-rich elements (AREs). Similarly to other cytokine mRNA species, GM-CSF mRNA contain AU-rich sequences in the 3' non-coding region that confer transcript instability (49). This was clearly shown by Shaw and Kamen (366) when a stable β-globin mRNA was rendered unstable when its 3' UTR was replaced with GM-CSF multiple ARE-containing 3' UTR. The mechanisms leading to transcript instability and a short half-life appear to be the result of recognition and binding of specific ARE-binding proteins, like the zinc-finger protein tristetraprolin (367). In contrast, the presence of activating p38 mitogen activated protein kinases (MAPK) leads to phosphorylation of tristetraprolin, decreased binding to ARE-recognition sequences, and overall increase in GM-CSF mRNA stability (368, 369). It therefore appears that the production of GM-CSF and other cytokines is dependent not only on an increased rate of transcription, but also on the activation of concurrent mechanisms aimed at promoting mRNA stability.

3.4.1. The GM-CSF receptor

All of the biological effects of GM-CSF are mediated via the GM-CSF receptor, a member of the gp140 family of the type I cytokine receptor group. In contrast to the homodimeric receptors of M-CSF and G-CSF discussed above, the GM-CSF receptor is composed of two distinct chains, α and β. The α chain is the primary binding chain that associates with GM-CSF with low affinity (Kd 1-10 nM) and rapid dissociation kinetics (334, 370, 371). The β chain, although it does not display intrinsic binding affinity for GM-CSF alone, is necessary for signal transduction and mediates the high affinity binding of GM-CSF together with the α receptor (Kd 30-100 pM) in a complex that displays slow dissociation kinetics (371-374).

GM-CSF receptors are primarily expressed, albeit in low numbers (~50-500), in human and murine macrophage, neutrophil, and eosinophil lineages (5, 375). GM-CSF receptors are also expressed on CD34$^+$ progenitor cells (both Lin$^+$ and Lin$^-$), erythrocyte and megakaryocyte precursors, B and T fetal lymphocytes, vascular endothelial cells, fibroblasts, osteoblast-like cells, and uterine cells (376-381). Maturation of hematopoietic cells along the various lineages differentially affects their expression of GM-CSFR subunits; for example, a recent study found that the βc subunit was expressed at very low levels in quiescent hematopoietic progenitor cells. The receptor expression increased following their initial differentiation along either the erythroid, megakaryocytic, granulocytic, or monocytic lineages, and only continued increasing in the granulocytic and monocytic lineages in subsequent differentiation stages, whereas it declined to undetectable levels in both the erythroid and megakaryocytic lineages (382). This correlates with the observed GM-CSF-mediated induction of growth, differentiation, and survival of macrophage, granulocyte, erythrocyte and megakaryocyte cells from bone marrow progenitors, as well as the modulation of functional activities in mature effector cells such as neutrophils, macrophages and dendritic cells. GM-CSF receptors have also been detected in several primary myeloid leukemic cells, some lymphoid leukemic cells, and several non-hematopoietic tumor cell lines (383-387). However, the significance of the GM-CSFR in non-hematopoietic tumor cell lines remains unclear.

3.4.1.1. The α-chain of GM-CSF receptor

The human GM-CSF receptor (GM-CSFR) α-chain (CD116) is encoded by a gene located in the pseudoautosomal region (PAR) of the X and Y chromosomes at Xp22.32 and Yp11.3, respectively, and spans an area of about 45 kb (370, 388). This region is localized at the tip of the short arms of the sex chromosomes, within 1,180-1,300 kbp of the telomere and 190 kbp from the gene encoding for the α-chain of the IL-3 receptor (389-391). In contrast, the murine GM-CSFR α-chain gene maps to the telomeric band D2 on the mouse autosomal chromosome 19 (392). The positioning of the GM-CSFR α-chain gene in the mouse appears to have resulted from GM-CSFR α-chain gene transfer from the X chromosome to chromosome 19 after branching off from the human lineage (393). The close proximity between the GM-CSFRα and IL-3Rα genes in humans does, however, point to a common origin between these two genes. In fact, recent studies indicate a close relationship between the genes for IL-3Rα, IL-5Rα, and GM-CSFRα: they all seem to have arisen from a common autosomal ancestral gene that was translocated to the eutherian X chromosome after the marsupials' divergence about 130 million years ago (394-396). The IL-3Rα and GM-CSFRα genes, however, share a closer evolutionary relationship as they contain an additional intron in their C domain that is not present in the IL-5α gene.

Analysis of the 5'-flanking region of the GM-CSFR α-chain gene shows the presence of several putative transcription regulatory sites. These include putative binding motifs for several transcription factors including PU.1, AP-1, GATA, c-Ets, SP1, C/EBPs, and NK-κB (397). The region between nucleotides –48 and –29 shows over 75% homology with a PU.1 binding site-containing region in the IL-3R α-chain gene promoter, consistent with the close evolutionary relationship between these two genes. Interestingly, 5' deletion mutants of both promoters indicated that this PU.1 site only plays an important role in the activation of the GM-CSFR α-chain gene; this points to the development of alternate mechanisms of transcriptional regulation for these two genes that may explain, at least in part, the differences in the cells targeted by these two colony-stimulating factors.

Molecular analysis of the full-length cDNA encoding the human and mouse GM-CSFR α-chains has shown that they encode for single transmembrane polypeptides of 400 and 387 amino acids, respectively, with limited homology (35%) at the amino acid level (371). Post-transcriptional modification of the human GM-CSFR α-chain leads to the formation of different mRNA species by alternative splicing. The predominant full-length form encodes for a type I transmembrane protein of 80kDa (370). Cleavage of the 22 amino acid signal peptide yields a mature polypeptide of 378 amino acids containing a 298 amino acid extracellular domain, 26 amino acid transmembrane domain, and a short cytoplasmic domain of 54 amino acids. The extracellular domain consists of an N-terminal region of about 100 amino acids that share significant sequence homology to similar regions in the IL-3 and IL-5 receptor α-chains, followed by a cytokine receptor domain containing four conserved cysteine residues and a WSXWS motif common to other members of this receptor family (398). The extracellular domain contains 11 potential N-glycosylation sites, and the presence of N-linked oligosaccharides is essential to both ligand binding by the α-subunit as well as signaling by the GM-CSF receptor (399). The cytoplasmic domain contains a short proline-rich motif similar to Box1 of the common β-chain, but displays no intrinsic enzymatic activity (400). Alternative splicing of the GM-CSF α-chain gene results in the formation of a second transcript where the C-terminal 25 amino acids have been replaced with 35 new amino acids (401). This form of the polypeptide is functional but is10-fold less abundant than the aforementioned form in the bone marrow and placenta. Alternative splicing at the 3' end of the GM-CSFR α-chain mRNA has also been shown to yield truncated polypeptides that lack both the transmembrane and cytoplasmic domains. The resulting transcripts encode for putative soluble forms of the α-subunit of the GM-CSF receptor and have been identified from a number of sources including human placenta (402), chorio-carcinoma cells (403), and juvenile myelomonocytic myeloid leukemia peripheral blood mononuclear cells (404, 405). Finally, two 5' alternatively spliced forms have also been identified in primary hematopoietic cells, blasts from acute myeloid leukemia patients, and several malignant cell lines (406). The partial or complete deletion of exon 2 sequences resulted in changes in the translation efficiency of the transcripts and may play a role in the regulation of GM-CSFR α-subunit expression.

3.4.1.2. The β-chain of GM-CSF receptor

The β-chain of the GM-CSF receptor is shared with the receptors for IL-3 and IL-5; it has the dual function of converting the initial cytokine binding to the α-subunit into a high-affinity state, and being the major signal transducer subunit of the receptor complex. In humans, this common β subunit (βc) is encoded by a single gene located on chromosome 22q12.2-13.1 (407). Analysis of the βc-chain proximal promoter indicated that Ets family members, including PU.1, are involved in the regulation of its basal expression (408). Moreover, additional characterization of upstream sequences indicated the presence of putative binding sites for several hematopoietic specific transcription factors (GATA, c/EBP, Myb, MZF), general transcription factors (cEts, Sp1, AP-1), and inducible transcription factors (STATs, NFκB). Although the specific events mediating βc chain gene expression remain largely obscure, these putative binding sites may help explain the up-regulation of βc gene expression in response to interferon-γ, erythropoietin, stem cell factor, IL-3, and tumor necrosis factor-α (409-412).

In contrast to humans, the mouse contains two closely related genes AIC2A and AIC2B that encode for an IL-3-specific β-subunit (βc_{IL-3}) and a βc-subunit shared between the GM-CSF, IL-3, and IL-5 receptors, respectively. Both genes are closely linked on the mouse chromosome 15, consist of 14 exons, and span an area of about 28 kb each (413). Despite having 91% sequence identity at the amino acid level βc_{IL-3} only binds IL-3 with low affinity and does not form a high affinity receptor complex with IL-5Rα or GM-CSFRα (408). The structural and sequence similarities between AIC2A and AIC2B genes, which also includes 90% identity at intronic sequences and 95% identity at 5' upstream sequences, originally led to the speculation that both genes had resulted from gene duplication after the divergence of mice and humans (413). More recently, however, sequences highly homologous to the 5' region of the human βc gene have been identified downstream from it, suggesting that the gene-duplication event may have occurred prior to the divergence between mouse and human; subsequently, the two genes would have evolved into βc and βc_{IL-3} in the mouse, whereas chromosomal mutations and gene reorganizations may have destroyed one of the genes in humans (408). In the rat only one transcript has been identified; it is homologous to the human βc chain functional mRNA species and the one derived from the murine AIC2B gene (414). In addition, β-chain sequences have been deposited in GenBank for guinea pig (Logsdon, N.J., Graham, A. and Scott, C.W.; unpublished observations) and *Xenopus* (Strausberg, R; unpublished observations).

Molecular analysis of the cDNA encoding for the human βc subunit predicts a mature 880 amino acid 95 kDa polypeptide possessing 3 potential N-glycosylation sites (29, 373). A similar βc subunit showing 55% amino acid identity to that of humans has been identified in mice (373). The extracellular portion of the receptor contains duplicate hematopoietin domains (also referred to as cytokine receptor modules, CRM) that represent conserved 200-amino acid domains of the cytokine receptor superfamily members including growth hormone, prolactin, Epo, Tpo, oncostatin M (OSM), cardiotropin, IL-2 (β- and γ-chains), IL-3 (α-chain), IL-4, IL-5 (α-chain), IL-6 (α-chain), LIF α-chain, gp130, ciliary neurotrophic factor (CNTF), IL-7, IL-9, IL-11 (α-chain), IL-12 (p40 subunit), IL-15 (β- and γ-chains), the recently described leptin receptor, and as described above the G-CSFR and the GM-CSFR α-chain (415, 416). Each hematopoietin domain is characterized by a number of conserved residues including: four cysteine residues in the membrane-distal portion of the domain which form two disulphide bonds; a Pro-Pro pair, which divides the domain into two 100 amino acid fibronectin type III-like subdomains, each of which displays a seven-stranded β-barrel structure (designated A-G); and the highly conserved membrane-proximal WSXWS motif. The extracellular portion of the β-chain is associated with the cellular membrane through a single transmembrane domain, which is followed by a 432 amino acid cytoplasmic domain, significantly longer than that of the α-chain but still displaying no intrinsic enzymatic activity.

Two isoforms of the human βc chain have been identified: the full-length transcript described above and an alternate form (β_{IT}) containing a 104bp deletion immediately 3' of the transmembrane region (417). This deletion results in the formation of a truncated polypeptide that conserves the 23 βc amino acids in the membrane-proximal cytoplasmic region and possesses 23 new amino acids in the C-terminal tail. This 46 amino acid cytoplasmic domain is significantly shorter than that of the full-length transcript and only retains the capacity to bind JAK2. It is, however, unable to transduce any mitogenic signals in response to GM-CSF, acting as a negative inhibitor of βc signaling. β_{IT} comprises about 20% of the β chain message in primary myeloid cells, but its proportion compared to βc is significantly reduced in response to GM-CSF or M-CSF (418). In contrast, β_{IT} is significantly up-regulated (90%) in blasts from acute leukemia patients suggesting a potential role in the pathogenesis of the disease.

3.4.2. Signal transduction and GM-CSF receptor

Interaction of GM-CSF with the low affinity α-chain of the GM-CSF receptor leads to a high affinity association through the formation of the α-/βc-subunit complex, and the subsequent transduction of intracellular signals that lead to proliferation, differentiation, survival, and activation of hematopoietic cells, predominantly in the macrophage and neutrophil lineages. Complex formation involves specific interactions, as those described above, between the GM-CSF ligand and both α- and β-receptor chains that appear to mediate specific conformational changes required for receptor activation (419-421). The stoichiometry of the active receptor complex remains unclear but there is evidence for the simple $\alpha_1\beta_1$ heterodimer structure as well as that of higher-order complexes such as $\alpha_2\beta_2$ tetramers that are stabilized by intersubunit disulphide linkages. The short α chain cytoplasmic domain is largely unable to signal in the absence of βc; however, it is required for receptor activation and overall signaling, presumably through stabilization of the α chain:βc complex (421). Different regions in the long 450 amino acid cytoplasmic βc subunit domain contribute to multiple functions; essential regions for the modulation of cell proliferation, differentiation, and survival pathways have been characterized (422, 423). The membrane-proximal domain has been identified to be required and sufficient for proliferation (424). It is known to bind members of the JAK kinase family, and is essential for the induction of STAT5, c-myc, pim-1, and cis (425, 426). In contrast, membrane-distal domains have been shown to be responsible for the major tyrosine phosphorylation of proteins, to promote c-fos and c-jun transcription, the induction of the Shc-Ras-MAPK and phosphatidylinositol 3-kinase (PI3K) pathways, and prevention of apoptosis (424, 426-428). Membrane-distal domains have also been implicated in the induction of differentiation and the negative regulation of receptor signals (423, 429).

Full receptor activation following oligomerization promotes intracellular signaling, but neither the α- nor the β- subunit cytoplasmic domains are known to possess intrinsic enzymatic activities. Instead, all downstream signaling events appear to require the association of cytoplasmic proteins with tyrosine kinase activity (416). These include members from the Janus kinase family, whose constitutive association with the Box1 membrane-proximal region of the receptor's cytoplasmic domain is augmented following ligand:receptor complex formation resulting in cross-phosphorylation. The exact mechanism resulting in JAK activation following receptor oligomerization, however, remains uncertain. Activated JAKs, particularly JAK2, subsequently initiate tyrosine phosphorylation on specific receptor cytoplasmic domain residues, providing docking sites for a variety of Src homology 2 (SH2) domain-containing proteins, including cytoplasmic signaling proteins like Shc and STATs, activating downstream signaling cascades (8, 422, 428). The wide array of signaling pathways induced by the GM-CSFR, however, is likely not limited to JAK-mediated activation as many other kinases are also known to be rapidly activated in response to GM-CSF and IL-3 including Src (8), Tec

(430, 431), Syk (432), Btk (433, 434), Lyn (435-437), Yes (437), FAK (438), Fyn (436), Fes (439), and Hck (436, 440).

3.4.3. Biological activities of GM-CSF

The granulocyte-macrophage colony-stimulating factor (GM-CSF) has pleiotropic and widespread effects on hematopoietic cells, and exhibits overlapping activities on hematopoietic progenitors with other cytokines including M-CSF, G-CSF, IL-3, IL-6, and SCF (415). It functions to promote the proliferation and maturation of neutrophils, eosinophils, and macrophages from bone marrow progenitors. It also acts as a growth factor for erythroid and megakaryocyte progenitors in synergy with other cytokines. Despite the pleiotropic nature of GM-CSF, however, significant specificity can be achieved, for example, through the relative concentration required for different responses. GM-CSF-mediated increase in cell viability occurs at significantly lower concentrations than those required for stimulation of cell proliferation, approximately 1/100 for hematopoietic progenitors *in vitro*. The role of GM-CSF in cell survival results from apoptosis inhibitory mechanisms (441). Conversely, withdrawal of GM-CSF *in vitro* from GM-CSF-dependent cells or normal hematopoietic progenitor cells leads to a significant decrease in cell viability (442, 443).

The effects of GM-CSF on cellular growth are also concentration-dependent, and are tightly linked to the identity of the responding cell and its stage of differentiation. For example, whereas murine GM-CSF provides appropriate stimulus for the development of granulocytic, macrophage and granulocyte-macrophage colony formation at concentrations between 2-80 pg/ml, higher concentrations are needed to stimulate eosinophil colony formation, and up to 10-fold greater concentrations are required to stimulate megakaryocyte, erythroid, and mixed progenitor (CFU-GEMM) colony formation (415). GM-CSF also displays combinatorial interactions with several other humoral factors, which may significantly elevate or decrease cell growth in the presence of varying amounts of the cytokine. These complex interactions can be illustrated, for example, by granulocyte-macrophage progenitors grown in the presence of GM-CSF and/or M-CSF. When these bipotential progenitors were treated with high concentrations of GM-CSF alone *in vitro* granulocyte colonies developed (444). In contrast, treatment with low concentrations of GM-CSF alone resulted in the formation of macrophage colonies. Furthermore, combinations of GM-CSF and M-CSF also differentially regulate the growth of bone marrow progenitors. Whereas M-CSF stimulation combined with very small amounts of GM-CSF dramatically elevated macrophage colony production (445), higher concentrations of GM-CSF lead to inhibition of M-CSF-mediated macrophage growth (446). This may be explained in part by the down-regulation of M-CSF receptors in response to GM-CSF (447, 448).

In addition to its role in the up-regulation of hematopoietic development, GM-CSF has been shown to have a profound influence on the biological functions of neutrophils, eosinophils, basophils, macrophages, lymphocytes, as well as endothelial cells, mesenchymal cells, and a variety of tumor-derived cells (216, 415, 449-451). These responses are widespread and point to a central role of GM-CSF in inflammation both through the direct activation of effector cells alone or in combination with other cytokines, as well as indirectly, through the stimulation of additional inflammatory mediator production. Some of these biological effects include enhanced antigen presentation, chemotaxis, synthesis of a variety of soluble mediators and enzymes, release of reactive oxygen intermediates and histamines, antibody-dependent cell killing, and phagocytosis which contribute differentially to the immune defenses against bacterial, viral, fungal, and parasitic infections as well as tumor development.

As a result of its role in the up-regulation of hematopoietic development as well as the stimulation of effector cell functions, the potential role of GM-CSF in clinical applications has been examined. Early experiments indicated that GM-CSF administration to mice, dogs, and primates largely paralleled *in vitro* observations (450). GM-CSF administration was primarily associated with an increase in circulating neutrophils, although this effect was not as pronounced as when G-CSF was used. GM-CSF stimulation also leads to an increase in monocytes, eosinophils, and lymphocytes, and may also have contributed to platelet production, although the effects on this group were variable. GM-CSF rapidly primed mice for enhanced proinflammatory cytokine production in response to LPS and TNF-α (452), and increased the capacity of granulocytes to produce superoxide as well as monocyte cytotoxicity in human donors (453). As a result GM-CSF has been applied clinically, for example, in the recovery from chemotherapy-induced neutropenia, in the up-regulation of hematopoiesis following bone marrow transplantation, in the treatment of fungal infections, and in chemo-immunotherapeutic applications against a variety of tumors (454-461). However, GM-CSF has also been implicated in disease progression. For example, it has been associated in the pathogenesis of Rheumatoid Arthritis (RA); elevated levels of GM-CSF in the synovial fluid of RA patients leads to up-regulation of HLA-DR production, autoantigen presentation, and activation of T cell responses which are central to the development of synovial inflammation associated with the disease (462). GM-CSF also augments neutrophil mediated cartilage degradation leading to severe structural damage.

3.4.4. Negative regulation of GM-CSF activity

Up-regulation of GM-CSF production in response to a variety of cytokines, antigens, or inflammatory agents results in the stimulation of hematopoietic events in the bone marrow, increased mobilization of effector cells and their precursors (particularly of the macrophage and granulocyte lineages) to the peripheral blood, and the induction of functional responses in mature cells throughout the body. Following this increased production, as is the case with the other CSFs, GM-CSF activity can be efficiently regulated through a number of mechanisms. Its concentration in the bloodstream can be controlled through metabolic degradation in the liver and kidney. Intravenously injected radiolabeled GM-CSF, like M-CSF and IL-3, accumulates rapidly in the liver thus contributing to the short half-life of plasma GM-CSF (139, 463, 464). Unlike the situation with other CSFs, however, the kidney appears to have only a limited contribution to the metabolic degradation of GM-CSF; intravenously injected GM-CSF was detected, but only in low amounts, in the urine of mice. In addition, local GM-CSF activity can be effectively modulated through its consumption and degradation by receptor-mediated endocytosis, by complexing with secreted soluble receptors, or by direct inhibition of the signaling pathways triggered by receptor-ligand interactions.

3.4.4.1. Down-regulation of GM-CSF receptor signaling

The activation of JAK tyrosine kinases plays a central role in the activation of signaling pathways as a result of GM-CSF stimulation. It is, therefore, not surprising that several of the mechanisms aimed at controlling these responses result from the inhibition of JAK kinase activity. One mechanism that terminates JAK activation involves SHP-1, a tyrosine phosphatase containing an SH2 domain that has been described in the M-CSF and G-CSF sections. Recruitment of SHP-1 to the GM-CSF receptor complex leads to the association of SHP-1 with the tyrosine phosphorylated βc cytoplasmic domains, presumably at Tyr612, resulting in the subsequent dephosphorylation and inactivation of receptor kinases (465-467). Alternatively, SHP-1 can also bind directly to the JAK kinases, such as JAK1 or JAK2, via an SH2-independent mechanism (468-470). Consistent with these reports, bone marrow progenitors and mature immune cells from motheaten mice, harboring a mutation that inactivates SHP-1 activity, have been found to display enhanced proliferative responses to GM-CSF (471, 472). In addition to SHP-1, other phosphatases, including SHIP and pp135 appear to be responsible for the negative regulation of IL-3/IL-5/GM-CSF signaling, and although the exact mechanisms mediating these responses remain to be elucidated they also seem to control the level of ligand-induced phosphorylated substrates (473-475). Inhibition of JAK activity has also been shown to be mediated by members of the SOCS family, several of which have been shown to be up-regulated in response to GM-CSF stimulation (147, 148, 471, 476). SOCS proteins are known to interact with specific targets through their SH2 domains, inhibit JAK activity through their N-terminal domains, and induce bound protein degradation through association with the proteasome. The inactivation of JAK/STAT signaling may also be controlled by additional cytoplasmic agonists including the cytokine inducible SH2-containing protein [CIS; (477)], JAK-binding protein [JAB; (478)], and STAT-induced STAT inhibitors [SSI; (300)].

3.4.4.2. Internalization and degradation of ligand-receptor complexes

As with other CSFs, receptor-mediated consumption of GM-CSF and its receptor represents an effective mechanism of down-regulation of GM-CSF activity. Mature and immature myeloid cells readily decrease the levels of GM-CSF though receptor-mediated absorption and subsequent metabolism (290, 479). The rate of internalization increases proportionally following ligand binding and results in the preferential internalization of occupied receptors followed by rapid ligand degradation and release. A recent study

(480), appears to shed light both on the mechanism leading to receptor internalization as well as the refractory period to βc-engaging cytokines that follows activation with any of GM-CSF, IL-3, or IL-5. Martinez-Moczygemba and colleagues found that following stimulation with IL-5, IL-3, or GM-CSF (βc engaging cytokines) full-length βc expression was down-regulated resulting in a transient unresponsiveness to subsequent stimulation with either of these cytokines; βc levels were not reduced as a result of decreasing βc mRNA levels but from the direct proteasomal degradation of the βc cytoplasmic domain. This process involved: a) the formation of a high affinity ligand/α-chain/βc-chain receptor complex following stimulation with a βc engaging cytokine; b) the activation of intracellular signaling pathways following receptor activation and phosphorylation of specific residues in the cytoplasmic domain in the βc subunit; c) the rapid proteasome-mediated degradation to terminate agonistic signaling by elimination of phosphorylated βc residues, resulting in the formation of ligand/α-chain/βc-chain complexes where the βc cytoplasmic domain had been degraded; and d) the initiation of endocytosis and lysosomal degradation of the truncated complex. In addition, stimulation with a βc-engaging cytokine resulted in the selective down-regulation of mRNA levels of its corresponding α-chain, and the increased expression of a soluble form of the α -chain that bound ligand with similar affinity to that of the membrane-bound form (477). Therefore, this process has implications both for the heterotypic and homotypic desensitization of signaling by all of the βc-engaging cytokines. Moreover, the cellular desensitization to subsequent βc-engaging cytokine stimulation via this mechanism may also add an instructive component to the stochastic myeloid differentiation process particularly in the earlier stages of hematopoietic development.

3.4.4.3. Synthesis and release of soluble GM-CSF receptors

As mentioned above, soluble forms for the ligand-specific α-chain of βc-engaging cytokines have been described, and appear to provide an effective mechanism for the selective decrease of extracellular cytokine levels. For GM-CSF, specifically, transcripts encoding a soluble form of the ligand-binding α-subunit (sGM-CSFRα) have been detected in the bone marrow, monocytes/macrophages, chorio-carcinoma cells, rheumatoid synovial tissue, and rheumatoid synovial tissue T cell lines (403, 481).

Moreover, sGM-CSFRα transcripts were the predominant message in synovial fibroblasts and osteoarthritis synovial tissue when compared to the transmembrane form of the α-subunit. The transcript encoding sGM-CSFRα results from alternative splicing of the parental sGM-CSFRα gene product, and encodes a protein that is missing both the transmembrane and cytoplasmic domains, but retains the 295 amino acid ectodomain (402, 403, 482). Moreover, the alternative splicing event results in a frameshift that leads to the addition of 16 amino acids to the carboxy terminus of the sGM-CSFRα protein. Analysis of the recombinant sGM-CSFRα shows a heavily glycosylated 60 kDa protein that binds GM-CSF in solution with the same affinity as membrane-bound GM-CSFRα (Kd 2-10 nM) (370, 482, 483). Even though sGM-CSFRα can adopt oligomeric forms in solution, GM-CSF binding appears to be mediated by the monomeric form in a 1:1 configuration with its ligand (484). Exogenous sGM-CSFRα does not associate with βc in the presence or absence of GM-CSF, and antagonizes the function of GM-CSF *in vitro* by preventing its binding to the cell surface and the formation of high affinity GM-CSF receptors (482, 485, 486). The inhibition of GM-CSF activity could also be demonstrated by a direct block in the proliferation of bone marrow progenitors and several GM-CSF-dependent cell lines (403, 481, 482, 487). sGM-CSFRα protein has been detected in normal human plasma, normal human granulocytes and monocytes, as well as a human chorio-carcinoma cell line (JEG-3), and various human myeloid leukemic cell lines (U937, THP-1, and HL-60) (483, 488, 489). In monocytes, sGM-CSFRα secretion is up-regulated in response to GM-CSF and inflammatory mediators such as LPS, PMA, and calcium ionophore (483). However, it is important to note that sGM-CSFRα protein formation is mediated not only by generation of the sGM-CSFRα alternative spliced mRNA form, but may also develop through proteolytic cleavage of the membrane-bound GM-CSFRα parent form.

3.5. Multi-colony-stimulating factor (IL-3)

The constant turnover of blood cells requires the up-regulation of proliferation and differentiation events in the hematopoietic tissues resulting in the production of committed progenitors to each of the eight blood lineages. Interleukin-3 (IL-3) has the broadest target specificity of all the hematopoietic growth factors and plays a central role in the production of macrophages, neutrophils, eosinophils, basophils, mast cells, megakaryocytes, and erythroid cells through stimulation of the pluripotent hematopoietic stem cells and their derivatives (490).

The human IL-3 gene is located on chromosome 5 near segment 5q31, and contains five exons and four introns that span an area of approximately 3.2 kb (491). It is positioned in tandem to the gene for GM-CSF and in close proximity to the genes for IL-4, IL-5, IL-9, and the M-CSFR (312). The close proximity and similar structural organization of the IL-3 and GM-CSF genes appear to point to a common evolutionary origin (492). This is especially interesting considering a similar relationship has been suggested for the genes encoding the IL-3 and GM-CSF α-chains (see GM-CSF section). The full-length human IL-3 mRNA encodes a precursor protein of 152 amino acids, with a 19 amino acid hydrophobic leader sequence, one interchain disulphide bridge (Cys 16-84), and two potential N-linked glycosylation sites at Asn15 and Asn70 that yield a core protein of 14.6 kDa (493). Cleavage of the signal peptide yields a mature 133 amino acid IL-3 protein that is expressed as a protein species ranging in molecular weight from 14 to 30 kDa, presumably due to various degrees of N-glycosylation (29). In the mouse the IL-3 gene is located on chromosome 11 at bands A5 to B1, and displays structural characteristics similar to the human gene, including its close association with the GM-CSF gene and similar transcriptional regulatory elements (494). The full-length murine IL-3 mRNA encodes for a molecule of 140 amino acids containing a 26 amino acid hydrophobic leader sequence that, upon cleavage, results in the production of a core polypeptide with a molecular weight of 15 kDa, two intramolecular disulphide bonds (Cys 17-80 and Cys 79-140) of which only the former is crucial for biological activity, and four potential N-glycosylation sites at Asn16, Asn44, Asn51, and Asn86 (495-497). The native mouse protein has a molecular weight of 22-32 kDa, presumably due to various degrees of N-linked glycosylation; however, glycosylation is not required for biological activity. Human and murine IL-3 coding sequences only show 45% identity at the nucleotide level and 29% identity at the amino acid level and do not cross-react in their biological activities. As a result, following the initial characterization of the murine species it took several years before the identification of a human homologue. Other than human and mouse, sequences encoding for IL-3 from a variety of species have been identified including gibbon, chimpanzee, tamarin, marmoset, rhesus monkey, bovine, ovine, rat (493, 498, 499), and dogs (McSweeney, P.A., *et al*; unpublished observations). Even though these sequences are all derived from mammalian species significant variability is observed; only a few regions, likely important for biological activity, are conserved during mammalian evolution.

IL-3 is predicted to have a four α-helical bundle core structure similar to that of GM-CSF and IL-5, placing it in the short-chain α-helical bundle type I cytokine family (173, 500). Structure-function studies of human IL-3, by generation of interspecies chimeras and mapping of epitopes by using neutralizing monoclonal antibodies, have shown that two noncontiguous helical domains located near the NH_2-terminus (Pro33 and Leu34) and the COOH-terminus (Leu111) of the polypeptide are responsible for binding to the receptor and are necessary for biological activity (501-504). Other studies further indicated that Asp21 in the first α-helix is essential for binding to the IL-3R α-chain, whereas the adjacent Glu22 is important for binding to the receptor β subunit (505, 506).

The major physiological sources of IL-3 are activated T-lymphocytes (507-510). Mast cells have also been shown to produce IL-3 following IgE Fc receptor cross-linking or after co-culture with fibroblasts (359, 511, 512). In addition, there are reports of IL-3 production by other cells including NK cells (513), eosinophils (360), keratinocytes (514), microglial cells (515), thymic epithelial cells (516), and stromal cells (517). Regulation of IL-3 gene expression is achieved at the level of transcription as well as post-transcriptionally. At the transcriptional level IL-3 gene expression is modulated by multiple promoter and enhancer elements, many of which it shares with the GM-CSF gene (500, 518). These reside mostly in a 350 nucleotide region upstream from the transcriptional start site, and their importance is highlighted by the degree of conservation (59%) between murine and human sequences compared to the degree of homology between the IL-3 genes themselves (45%). The 5'-flanking region contains several potential binding sites for transcription regulatory elements including AP-1, AP-2, CK-1, CK-2, elf-1, ets-1, CBFα, CREB, Sp1, and NFAT-1. In addition, an inducible DNAse I hypersensitivity site exists between the IL-3 and GM-CSF genes that displays strong enhancer activity on both genes through interactions with AP-1 and NF-AT (519). Inhibitory elements can also be found in the IL-3 promoter region including a nuclear inhibitory protein (NIP) binding sequence located between base pairs –271 and –250 (520). Post-transcriptional regulation of IL-3 expression is mediated by the presence of AU-rich elements (AREs). Similarly to other cytokine mRNA species, IL-3 mRNA contain AU-rich sequences in the 3' non-coding region that confer transcript instability (49). These are located in a highly conserved region (76% between mouse and human) between the termination codon and polyadenylation signals (521). Similar to GM-CSF transcripts, the stability of IL-3 mRNA, through its AU-rich elements, appears to be directly related to independent turnover control by PI3-K and p38 MAPK pathways (522).

3.5.1. The IL-3 receptor

All of the biological effects of IL-3 are mediated via the IL-3 receptor, a member of the gp140 group of the type I cytokine receptor superfamily. In contrast to the homodimeric receptors of M-CSF and G-CSF and similar to the heterodimeric GM-CSF receptor discussed above, the IL-3 receptor is composed of two distinct chains, α and β. The α chain is the primary binding chain that binds IL-3 with low affinity (Kd 50-100 nM) and has rapid dissociation kinetics (5). The β chain, although it does not display intrinsic binding affinity for IL-3 alone, is necessary for signal transduction and mediates the high affinity binding of IL-3 together with the α receptor (Kd 100-300 pM) in a complex that displays slow dissociation kinetics.

The high-affinity IL-3 receptor has been detected on different hematopoietic cells including primitive and committed progenitor cells, granulocytes, monocytes/macrophages, megakaryocytes, and B-lymphocytes (523, 524). Within the primitive compartment it can be found in a subset of $CD34^+$ cells in the fetal liver, fetal bone marrow, cord blood, adult bone marrow, and mobilized peripheral blood. It does not, however, appear to be expressed in the most primitive cells of the $CD34^+$ subset or the newly identified primitive $CD34^-$ population. In addition, IL-3R expression has also been detected in a number of cells outside the hematopoietic system, including vascular and connective tissue-type cells like endothelial cells, smooth muscle cells, and fibroblasts (525). Although the role of IL-3 on these cells is largely unknown, IL-3 together with other inflammatory cytokines like TNF-α and IFN-γ may stimulate the expression surface molecules like E-selectin and IL-8 that may in turn facilitate neutrophil transmigration through the epithelium during inflammatory processes, as well as stimulate MHC class II expression and the secretion of early-acting hematopoietic cytokines like G-CSF and IL-6.

3.5.1.1. The α-chain of IL-3 receptor

The IL-3 receptor α-subunit (CD123) is a type I transmembrane glycoprotein belonging to the cytokine receptor superfamily, as are the related IL-5Rα and GM-CSFRα to which it displays 54% and 43% sequence homology, respectively (500). The human IL-3 α chain gene is located in the pseudo-autosomal region of the sex chromosomes at Yp13.3 and Xp22.3, and contains twelve exons and eleven introns that span an area of approximately 40 kb (390, 391). It is positioned in close proximity (190kbp) to the gene for the GM-CSFR α-chain and displays a similar structural organization, suggesting a common evolutionary origin for both genes (see GM-CSF section). In contrast the mouse IL-3Rα chain gene is located in the proximal region of chromosome 14, separated from the mouse GM-CSFRα gene, which is on chromosome 19 (526). Analysis of the 5'-flanking region of the human IL-3R α-chain gene shows the presence of several putative transcription regulatory sites. These include a typical TATA box (TATAAA) at position –35 and putative binding motifs for several transcription factors including SP1, PU.1, C/EBPs, AP-2, AP-4, and GATA-1 (397). The region between nucleotides –375 and –356 shows over 75% homology with a PU.1 binding site-containing region in the GM-CSFR α-chain gene promoter consistent with the close evolutionary relationship between these two genes. Interestingly, 5' deletion mutants of the IL-3Rα promoter revealed that this PU.1 binding site did not affect the promoter activity, unlike that of the GM-CSFRα gene where it plays a major role in the promoter's transcriptional activity (397).

The IL-3Rα gene encodes for a type I transmembrane protein of 41.3 kDa (527). Cleavage of the 18 amino acid signal peptide yields a mature polypeptide of 360 amino acids containing a 288 amino acid extracellular domain that plays a significant role in receptor binding to IL-3, a 20 amino acid transmembrane domain, and a short cytoplasmic domain of 52 amino acids. The extracellular domain consists of an N-terminal region of about 100 amino acids that share significant sequence homology to similar regions in the GM-CSF and IL-5 receptor α-chains, followed by a cytokine receptor domain containing four conserved cysteine residues and a WSXWS motif common to other members of this receptor family (398). The extracellular domain contains six potential N-glycosylation sites, and the presence of N-linked oligosaccharides is essential to both ligand binding by the α-subunit as well as signaling by the IL-3 receptor (521). N-glycosylation at these sites appears responsible for the observed molecular weight of 70 kDa. The cytoplasmic domain contains a short proline-rich motif similar to Box 1 of the common β-chain, but displays no intrinsic enzymatic activity. No alternative forms for the IL-3Rα chain have been described.

3.5.1.2. The β-chain of IL-3 receptor: Functional redundancy versus signal transduction specificity

The IL-3 receptor shares a common β chain with receptors for GM-CSF and IL-5. The specifics of gene structure and expression of this βc-chain have been described above in the GM-CSF section. An additional point needs to be considered: it is fairly obvious that sharing of the signal transducing β_c subunit between these receptors would allow for the significant functional redundancy observed, through the activation of overlapping signaling pathways. However, it is less obvious to determine how signal transduction specificity is achieved by these three ligands; despite the role that the α chain has in the stabilization of the activated high affinity receptor complex, it does not appear to contribute significantly to the induction of downstream signaling cascades *per se*.

Through the production of ligand-specific β chains: As described above, a second β chain is present in mice that associates exclusively with the IL-3 α chain (IL-3Rα), and is therefore called βIL-3. Both βc and βIL-3 subunits form functionally indistinguishable high-affinity IL-3Rs with the same IL-3R-specific α-subunit (528). However, βIL-3, unlike the βc chain, does not form a high affinity receptor complex with IL-5Rα or GM-CSFRα (408) and is therefore able to confer signal specificity upon IL-3 binding. Remnants of a gene encoding for an analogous chain in humans have been identified whose functionality appears to have been lost in evolution.

Through the hierarchical association between various cytokines to a common receptor: GM-CSF, IL-3, and IL-5 share a common β subunit for the formation of their high affinity receptors and subsequent activation of downstream signaling pathways. Generally, these receptors are differentially expressed among cells of the hematopoietic system (5). However, cross-competition between the cytokine ligands for the βc chains results in the formation of a hierarchy of βc binding in those cells expressing the appropriate α chains as well as the βc chain (529-531). GM-CSF shows the greatest ability to recruit βc, followed by IL-3 and IL-5. As such, affinity for the βc subunit may induce differential biological activities even in the presence of more that one of the cytokine ligands and/or their specific α-chains.

Through differential induction and/or integration of signaling pathways – the cellular background issue: As described above, β chains are widely expressed throughout the hematopoietic system. Despite them being able to associate with an array of adaptors and signaling molecules, it is important to recognize that the patterns of expression of these cytoplasmic proteins differs depending on the cell type, maturation stage, and the proliferation and/or activation signals that the cell is exposed to. As such, the end-result is intimately dependent not only on a functional receptor that can transduce extracellular signals, but also in the intracellular environment that must provide the necessary cytoplasmic signaling proteins to generate a downstream response. For example, the tyrosine kinase Btk, predominantly expressed in B cells, has been shown to associate to the βc cytoplasmic domain upon activation (see above). Whereas inactivation of Btk expression in X-linked immunodeficient mice leads to impaired IL-5 signaling in B cells, it does not affect normal eosinophilic IL-5 mediated development (433). RACK1, an adaptor protein found to associate with the βc subunit after IL-5 or TPA stimulation, is preferentially expressed in monocytes, eosinophils, as well as B and T lymphocytes but not in human neutrophils potentially leading to cell-type-specific activation of signaling pathways downstream of RACK1 (532).

The cellular background also has implications for the integration of signaling cascades at the level of gene expression. The differential expression of transcription factors among cells of the hematopoietic system (87) provides an elegant mechanism for the modulation of the genes and their levels of expression in different cells despite parallels in the cytokine ligands and the receptors being activated.

3.5.3. Biological activities of IL-3

Interleukin-3 represents one of a select group of cytokines, that also includes GM-CSF, stem cell factor (SCF or c-kit ligand), Flt-L (the ligand for the flt2/3 receptor), erythropoietin (EPO), and others, that promote the development of early hematopoietic cells into cells of the myeloid, lymphoid, and erythroid lineages (533). Among the colony-stimulating factors, IL-3 targets the earliest progenitors even though significant overlap exists with GM-CSF, which acts on a slightly more mature subset (534, 535). In colony-forming assays IL-3 supports colony formation by normal hematopoietic progenitors including multi-lineage (CFU-GEMM), granulocyte-macrophage (CFU-GM), granulocyte (CFU-G), macrophage (CFU-M), megakaryocyte (CFU-Mk), eosinophil (CFU-Eo), basophil (CFU-b), and in the presence of erythropoietin both in early (BFU-E) and late (CFU-E) erythroid colonies [reviewed in (521)]. Moreover, IL-3 synergizes with other cytokines including stem cell factor (SCF), IL-1, IL-6, IL-11 and G-CSF to promote cell growth in part through shortening of the G_0 period in the target cells (523, 536, 537). Other studies further support the role of IL-3 in the induction of proliferation, differentiation, and survival of early hematopoietic progenitors and their progeny (5, 490). At later stages of maturation, IL-3 growth-promoting activity appears to be more restricted to eosinophils, basophils, and perhaps a subpopulation of monocytes (500).

In vivo, administration of IL-3 also promotes up-regulation of hematopoietic events and results in the increased production of leukocytes, red blood cells, and platelets (538). IL-3 promotes the growth of multipotent stem cells able to reconstitute the hematopoietic system of irradiated animals (539). Furthermore, IL-3 over-expression induced in murine hematopoietic progenitors resulted in a fatal but non-neoplastic myeloproliferative syndrome (540). The functional significance of IL-3 and its receptor has also been investigated by several gene-targeting approaches. Mice lacking the IL-3R α-chain show hyporesponsiveness to IL-3, while other growth factors such as GM-CSF and G-CSF are able to stimulate normal colony formation (541); this is despite the expression of normal levels for the β subunit. IL-3Rα deficient mice, however, show no gross hematological abnormalities and effectively defend against bacterial and parasitic infections supporting the presence of significant functional redundancy with other cytokines. βc chain mutant mice also fail to show significant hematological defects; they exhibit normal numbers of hematopoietic stem cells and their progenitors in the bone marrow, but do show a reduced eosinophil count and a pulmonary alveolar proteinosis-like disease (542). βc-deficient mice are unable to respond to stimulation with GM-CSF and IL-5, but retain the ability to respond to IL-3, presumably through the action of the β_{IL-3} subunit. Compound knockout mice lacking both the IL-3 ligand and the βc subunit also gave similar results (541). These mice lacked both a functional IL-3 cytokine and functional receptors for IL-3, IL-5, and GM-CSF, due to a lack of the βc subunit. The double-mutant mice were apparently normal and fertile. They displayed normal blood cell numbers, except for reduced eosinophil numbers and a lack of eosinophilic response to parasites. They also displayed normal immune responses to *Listeria monocytogenes*, and normal hematopoietic recovery after administration of the cytotoxic drug, 5-fluorouracil. Overall, it appears that alternative mechanisms are able to compensate for the IL-3/IL-5/GM-CSF system in both steady-state as well as in emergency conditions.

3.5.4. Negative regulation of IL-3 activity

IL-3 is undetectable in the blood of normal animals (543); only in one report were levels of 25-40 pg found in 16% of the samples observed (544). This is in part due to the short half-life of IL-3 in circulation. Interleukin-3 intravenously injected into mice was degraded in about 20 minutes (545). This clearance occurs mainly through the kidney, with most of the filtered IL-3 resorbed and destroyed in the renal tubules with only degraded material passing through into the urine (490). A significant portion of injected IL-3 also accumulates in the liver where it appears to be cleared through the action of the parenchymal cells (5). In addition to these metabolic degradation mechanisms, receptor-mediated internalization and degradation of this CSF appears to contribute to the negative regulation of IL-3 activity. *In vitro* experiments indicate that this internalization process occurs rapidly and is followed by a fairly rapid intracellular degradation process (529, 546).

The dominant role that the βc subunit plays in the transduction of IL-3, GM-CSF, and IL-5 signals leads to a significant overlap between the mechanisms aimed at inhibiting the activation of downstream signaling cascades [see GM-CSF section and (8, 533)]. Although it is expected that additional inhibitory mechanisms may display specificity by associating with ligand-specific α-chain cytoplasmic domains or the mouse IL-3-specific β_{IL-3} subunit, none of these mechanisms have yet been characterized. Moreover, in contrast to GM-CSF, G-CSF, and now M-CSF no soluble receptors have been described for IL-3 that may inhibit signaling cascades through association with the ligand prior to its binding to the membrane-bound receptors.

3.6. Concluding remarks

From the literature, there is convincing evidence that the development of hematopoietic cells from early progenitors to mature blood cells, particularly for those in the myeloid lineage, is mediated by the colony stimulating factors. These hematopoietic regulators carry instructive signals for proliferation, differentiation, survival, and perhaps an instructive component of cellular commitment. CSFs, however, do not act alone. They are part of a well-orchestrated network of intrinsic and extrinsic modulators that together guide a pluripotent stem cell to develop along one of the eight hematopoietic lineages. This network derives from the hematopoietic microenvironment that mediates the cell-cell interactions, cell-extracellular matrix interactions, and the exposure to cytokines that have been produced locally or arrived through the circulation. These cytokine gradients include the CSFs, but may also involve association with other cytokines like IL-6, LIF, oncostatin M, or IL-11 specialized for cellular differentiation control, as well as several inhibitors of hematopoietic development like TNF, TGFβ1, IFNs α/β and γ, IL-4, IL-10, or IL-13 (547). This network also involves intrinsic mechanisms, including stochastic events, as well as the transcriptional regulatory machinery that integrates extrinsic and intrinsic signals to modulate the pattern of gene expression culminating in an overall biological response.

The central role that the colony-stimulating factors play in the regulation of hematopoiesis and immune competence has led to the detailed characterization of their gene and protein structural determinants, as well as characterization of their interactions with other components from the network of hematopoietic regulators. These studies have been largely undertaken in mammalian systems, and suggest that there is significant functional conservation of CSF activities, even though there appears to be limited conservation in their gene and protein sequences. It is therefore not surprising that no colony-stimulating factor homologs have yet been identified in lower vertebrates. In contrast, there has been significant progress in the identification of CSF receptor homologs from a variety of lower vertebrate species, as described above. This may be associated with a higher degree of evolutionary conservation among CSF receptor sequences and/or the greater stability of their mRNA species in the absence of AU-rich destabilizing elements. If this continues to be the case despite improvements in molecular techniques, alternative approaches to the direct identification of CSFs solely based on sequence homology may prove more fruitful in the long run. These approaches may involve the use of the intrinsic ability of CSF receptors to bind their ligands, and will certainly be facilitated by the protein expression of various CSFR sequences that have already been identified. The challenge will lie in the establishment of effective methodologies aimed at raising preparations with high specific activity of the putative colony-stimulating factors and their subsequent efficient purification.

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CHAPTER II: GENERAL METHODS AND MATERIALS

1. CELL CULTURE

1.1. Fish

Goldfish (*Carassius auratus* L.) and carp (*Cyprinus carpio*) 10 to 15 cm in length were purchased from either Ozark Fisheries Inc. (Southland, MI) or Grassy Forks Fisheries (Martinsville, IN), or generously donated by Dr. Norman Stacey (Department of Biological Sciences, University of Alberta). Fish were maintained in the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were held at 20°C in a flow-through water system on a simulated natural photoperiod, as that of Edmonton, Alberta, for the duration of the experiments. The fish were fed to satiation daily with trout pellets. Fish were acclimated to this environment for at least 3 weeks prior to use.

1.2. Fish serum

Fish serum was obtained by bleeding common carp (15-40 cm). The fish were anaesthetized using tricaine methanesulfonate (TMS; approximately 40 mg l^{-1}; Syndel Laboratories) and bled by caudal venipuncture using a 1 ml syringe and a 23G needle. These fish were maintained solely for serum production and were bled every 8-10 weeks. Blood collected from these fish was allowed to clot at room temperature for approximately 1 hr and overnight at 4°C. The following morning blood samples were centrifuged at 1000x*g* for 20 min and serum removed. Serum was heat inactivated at 56°C for 30 min, filter-sterilized (0.22 µm), and stored at -20°C until used.

1.3. Culture medium and culture conditions

The culture medium used in all experiments was a modified form of that previously described for culturing a goldfish macrophage cell line [GMCL, (1)]. The formulation for the medium is listed in Table 2.1. Modifications to the original medium provided a powerful buffering capacity for culturing goldfish macrophages and leukocytes in medium exposed to air. Complete medium contained 100 U ml^{-1} penicillin

(Gibco), 100 μg ml^{-1} streptomycin (Gibco), 100 μg ml^{-1} gentamicin (Gibco), 10% newborn calf serum (Hyclone), and 5% carp serum. All goldfish cell cultures were grown at 20°C.

1.4. Isolation of goldfish kidney leukocytes

Leukocytes were isolated from the kidneys of goldfish using procedures described previously (2, 3). Fish were anesthetized with tricaine methanesulfonate (TMS; approximately 40 mg l^{-1}), and the kidneys aseptically removed and placed in ice-cold MGFL-15 medium. The kidneys were pressed through sterile stainless steel screens with medium containing antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin) and heparin (50 U ml^{-1}; Sigma). The cell suspension was layered on 51% Percoll (Pharmacia) and centrifuged at 400xg for 25 minutes. Cells at the medium-51% Percoll interface were transferred into clean tubes and washed twice by centrifugation at 200xg for 10 min at 4°C. The number of viable leukocytes was determined using the trypan blue exclusion method (Gibco). Typically, a yield of $1.5x10^7$ to $3.5x10^7$ kidney leukocytes was obtained per goldfish.

1.5. Production of mitogen-stimulated goldfish kidney leukocyte conditioned supernatants (MAF)

MAFs were raised by the mitogenic stimulation of mixed kidney leukocyte cultures, using procedures described previously (4, 5). Briefly, leukocytes from multiple fish were pooled, enumerated, and seeded at a density of $4x10^6$ cells ml^{-1} in 75 cm^2 tissue culture flasks. Cultures were allowed to equilibrate in complete medium (containing antibiotics) overnight. Cells were subsequently treated with concanavalin A (10 μg ml^{-1}, Boehringer Mannheim), PMA (10 ng ml^{-1}, Sigma), and calcium ionophore (100 ng ml^{-1}, Sigma) for 6 hr. Cultures were incubated in the presence of mitogens for 6 h, after which the mitogens and serum were removed by washing the cells with three exchanges of 25 ml Hanks balanced salt solution (1x HBSS). The remaining cells were resuspended in incomplete culture medium and incubated for 72 h at 20°C. MAF-containing supernatants were collected, filter sterilized, and stored at -20°C prior to use in the assays.

1.6. Establishment of primary kidney macrophage (PKM) cultures

PKM cultures were established by isolating goldfish kidney leukocytes using 51% continuous Percoll gradient centrifugation, as described above. Kidney leukocytes (KL) were subsequently cultured in 25 cm^2 tissue culture flasks containing 7.5 ml of complete medium and 25% filter sterilized GMCL-derived CCM or kidney leukocyte-derived CCM containing macrophage growth factor(s) (see below); cells were seeded at a concentration of $8.5x10^6$ cells $flask^{-1}$ from individual fish. Alternatively, KL were cultured in 75 cm^2 tissue culture flasks containing 15 ml of complete medium and 25% filter sterilized GMCL-derived CCM or kidney leukocyte-derived CCM; cells were seeded at a concentration of $18x10^6$ cells $flask^{-1}$ from individual fish. PKM were incubated for 6-9 days at 20°C before being used in assays, unless otherwise specified. Cell conditioned media (CCM) from these macrophage cultures were subsequently used as a source of macrophage growth factors (MGF) for establishing new PKM cultures.

1.7. Collection of cell-conditioned medium

Goldfish PKM cultures were established by stimulating hematopoeitic-derived kidney leukocytes with CCM containing MGF. Two types of CCM were used as sources of MGF in establishing macrophage cultures: 1) CCM obtained from 7-10 day old GMCL cultures (GMCL-derived CCM); and 2) CCM obtained from previous PKM cultures (PKM CCM), as described above. Cell conditioned supernatants were collected, centrifuged at 200x*g* for 10 min at 4°C, filter-sterilized (0.22 μm), and stored at 4°C until used.

1.8. Bioassay for macrophage growth factor activity in GMCL-derived CCM

Individual wells of a 96-well tissue culture plate were seeded in quadruplicate with 6.25, 12.5, 25, or 50 $x10^3$ kidney leukocytes and induced to proliferate by the addition of varying amounts (v/v) of CCM obtained from 7-to-10 day-old GMCL cultures (0, 6.25, 12.5, or 25% GMCL-derived CCM). Total volume in culture wells was 100 μl. Plates were sealed with Parafilm® and incubated for 8 days at 20°C. Medium was replaced after 4 days of cultivation by gently removing 50% (50 μl) of the medium from each well and replacing it with an equal volume of fresh medium containing the appropriate concentration of GMCL-derived CCM. The medium in the cultures was also changed 12 hr before the assessment of viability using the XTT assay described below.

The kinetics of MGF-induced proliferation of progenitor cells was determined by placing 6.25×10^3 or 2.5×10^4 leukocytes into wells of 96-well plates and culturing cells in the absence or presence of GMCL-derived CCM (final concentration of 25% v/v). The medium in cell cultures was replaced every 4 days (as described above) during the first 8 days of cultivation. Medium was subsequently replaced every 2 days for the duration of the experiment. Cell viability was determined at 0, 4, 8, 12, or 16 days of cultivation using the XTT viability assay.

1.9. Bioassay for macrophage growth factor activity in PKM-derived CCM

To demonstrate endogenous production of a MGF by kidney leukocytes, leukocytes were seeded in quadruplicate at a density of 1×10^5 cells/well in 96-well culture plates and the kinetics of growth determined at 0, 2, 4, 8, and 12 days after cultivation using the XTT viability assay (described below). Fresh medium was replaced after 4 days of cultivation and every 2 days thereafter. Supernatants from 0, 2, 4, or 8-day old cultures were removed and tested for their ability to restore the proliferative capability of kidney leukocytes seeded at 1.25×10^4 kidney leukocytes well^{-1}. Thus, 1.25×10^4 kidney leukocytes were seeded in quadruplicate in wells of a 96-well culture plate in a volume of 75 µl, and induced to proliferate by the addition of 25 µl of conditioned supernatants from the cultures seeded at 1×10^5 cells well^{-1}. Cell number was determined after 12 days of cultivation using XTT viability assay.

1.10. XTT proliferation assay for macrophage growth factor (MGF) activity in PKM cell conditioned medium

The macrophage growth-inducing activity of several PKM cell conditioned medium samples was assessed by seeding kidney leukocytes in triplicate at a density of 2.5×10^4 cells per well in 96-well culture plates (Falcon). The low seeding inoculum was chosen to minimize the effect of endogenous MGF production, thus maximizing MGF effect from added CCM samples. Cells were seeded in 75 µl of complete culture medium, and induced to proliferate by the addition of 25 µl of cell conditioned supernatants.

The number of cells in culture wells was indirectly determined using the XTT tetrazolium salt viability assay after 8 days of cultivation (6, 7). Viable goldfish macrophages reduce this compound to a soluble formazan in the presence of an electron coupler (e.g. CoQ or PMS) with the amount of reduction directly proportional to the number of viable cells in culture (Fig. 2.1). Briefly, 50 µl of Dulbecco's phosphate-buffered saline (D-PBS; Gibco BRL) containing D-glucose (1 mg ml^{-1}; Anachemia), XTT (1 mg ml^{-1}; Sigma), and co-enzyme Q (CoQ; 50 µg ml^{-1}; Sigma) or phenazine methosulfate (PMS; 0.383 mg ml^{-1}; Sigma) were added to the individual wells and incubated at 20°C for 2 or 18 hours, respectively. Optical densities were determined at 450 nm using a microplate spectrophotometer (Biotek).

2. MORPHOLOGICAL AND CYTOCHEMICAL ANALYSES

Microscopic examination of PKM cell morphology was performed after staining cytocentrifuge smears (Cytospin II, Shandon Instruments) with a modified Wright's stain (Fisher). Cytochemical characterization was achieved by staining PKM cytocentrifuge smears for non-specific esterase (using α-naphthyl acetate esterase), acid phosphatase, and myeloperoxidase, at 32°C according to the manufacturer's protocols (Sigma).

3. FUNCTIONAL ANALYSES

PKM were seeded into wells of a half area 96-well culture plate (2.5 x 10^4 cells well^{-1}) and stimulated with MAF (1:4 final dilution) and/or LPS (1 µg ml^{-1}, *Salmonella typhimurium*, Difco). The production of both reactive nitrogen and oxygen intermediates was determined.

Reactive nitrogen intermediates were measured by sampling culture supernatants for the presence of nitrite, using the Griess reaction (8-10). Briefly, 75 µl of macrophage supernatants were removed from individual wells and placed in a separate 96-well microtitre plate. One hundred microlitres of 1% sulfanilamide (Sigma) in 2.5% phosphoric acid (Anachemia) was added to each sample, followed by 100 µl of 0.1% N-naphthylethylene diamine (Sigma) in 2.5% phosphoric acid. Optical density was determined using a spectrophotometer (Biotek) at 540 nm. The molar concentration of nitrite in samples was determined from standard curves generated using known concentrations of sodium nitrite dissolved in medium (Fig. 2.2).

Production of reactive oxygen intermediates was done by measuring respiratory burst response of MAF and/or LPS-primed macrophages. PKM were seeded into wells of a half-area 96-well culture plate (2.5 x 10^4cells $well^{-1}$) and primed with MAF (1:4 final dilution) and/or LPS (1 μg ml^{-1}) for 6 or 48 hrs prior to measuring their respiratory burst response. Respiratory burst responses were assayed using the NBT method (11). Nitroblue tetrazolium (NBT; Sigma) was dissolved in dimethyl sulfoxide (20%w/v) to which pre-warmed (50°C) Dulbecco's PBS (Gibco) was added to completely dissolve NBT (final NBT concentration= 2 mg ml^{-1}). This NBT solution was heated for an additional 10 min at 50°C and subsequently filter-sterilized. PMA was added to the NBT solution such that the final triggering concentration in macrophage cultures would be 50 ng ml^{-1}. Fifty μl of the NBT/PMA solution was added to macrophage cultures and incubated for 25 minutes at room temperature. Supernatants were subsequently removed and macrophage layers fixed by adding 150 μl of 70% methanol for 1 min. Unreduced NBT was removed by washing cells several times with 70% methanol. Reduced NBT was dissolved by adding 60 μl of 2M KOH to individual wells and pipetting vigorously. Seventy μl of dimethyl sulfoxide (DMSO; Caledon) was then added to wells and optical densities read at 630 nm on a microtiterplate spectrophotometer.

The phagocytic ability of PKM was determined using the bacterium *Aeromonas salmonicida*. Bacteria were washed three times in serum free medium by centrifugation (10,000xg, 10 min) prior to addition to macrophages. PKM (5x10^5 cells) were placed into 6 ml polypropylene tubes (Falcon) in serum free medium and infected with bacteria at a multiplicity of infection of 10:1 (total volume of bacteria and cells was 500 μl in serum free media). Cells were allowed to phagocytose bacteria for 1 hr at 20°C. Non-phagocytosed bacteria were removed by centrifuging the cell suspension three times at 50xg for 10 min. The washed cell pellet was re-suspended in 500 μl of serum free medium; smears were made by cytocentrifugation (Shandon) and stained using a Modified Wright's stain (Fisher).

4. FLOW CYTOMETRIC ANALYSES

4.1. Preparation of fluorochrome-labeled cell suspensions

PKM were labeled with PKH26 using a modification of the protocol provided by the manufacturer (Sigma). Briefly, cells were concentrated by centrifugation, resuspended, and washed twice in serum free medium. The number of viable cells was determined using a trypan blue exclusion assay, enumerated using a haemocytometer, and resuspended in serum-free medium at a concentration of 5×10^6 cells ml^{-1}. Cells were labeled with PKH26 (4×10^{-6}M) at 25°C for 3 minutes. An equal volume of calf serum was added to stop the labeling reaction, and the labeling solution was removed by centrifugation. Finally, the cells were washed twice in complete medium by centrifugation and resuspended to the desired concentration. All centrifugation steps were done at 150xg for 10 minutes at 4°C.

4.2. Flow cytometric analyses of macrophage subpopulations

PKM were analyzed by flow cytometry using a FACScalibur flow cytometer equipped with a cell sorter (Becton Dickinson). Forward light scatter (size), side scatter (internal complexity), and fluorescence (FL2-H, excitation:488nm / emission:585nm) parameters were considered. All flow cytometric analyses were done on non-adherent PKM suspended in phosphate-buffered saline (PBS), unless specified otherwise. This minimized cellular damage sometimes associated with the trypsinizing protocol on the adherent population, which we have determined to represent a minority of the cells within PKM cultures and to display a similar flow cytometric profile to that on the non-adherent population (see Fig. 3.7, Chapter III). Flow cytometer instrument settings were stored and held constant throughout all experiments. Settings for the flow cytometer were as follows: 1) forward side scatter - photodiode voltage set to E00, AmpGain set to 1.00, 2) side scatter - photomultiplier voltage set to 480 V, AmpGain set to 1.00, 3) FL2-photomultiplier voltage set at 490 V, AmpGain set to 1.00. Three approaches were used to study cell development: 1) PKH26 analysis was used to examine proliferation events and differentiation patterns of PKM cultures. 2) Propidium iodide staining was used for cell cycle analysis for the 3 different subsets of cells. The results of preliminary experiments showed that different cell fixation protocols required for propidium iodide staining significantly altered both forward and side scatter characteristics of PKM, making the propidium iodide staining unreliable for the cell cycle analysis of fish macrophages. 3) The relative rate of PKM migration through the various phases of development (*lag*, *proliferative,* and *senescence*) was also assessed using flow cytometry (discriminating parameters are described in detail in Chapter V). These complemented parallel observation of changes in culture phenotype (discriminating parameters also described in Chapter V).

4.3. Analysis of PKM cultures using the Cell Proliferation Model (Sigma/Verity)

PKH26-labeled primary kidney leukocytes were observed using the FACScalibur flow cytometer for several days post-labeling (refer to specific experiments for details on the period of observation) to determine the proliferation kinetics and path of differentiation taken by these cells as the culture developed. Single log integrated fluorescence (FL2-H) histograms were analyzed using the Cell Proliferation Model and ModFit software (Sigma/Verity). ModFit deconvoluted the raw histograms into peaks. Each peak represented a generation. This analysis was based on the observation that daughter cells arising as a result of cell division retain approximately half the parental cell's lipid membrane. As a result, the PKH26-derived fluorescence intensity was halved with each cellular division.

Fluorescence signals, collected using four log amplifiers, were converted to a linear scale of 256 channels. In this configuration every 19.19 channels represents a factor of 2 in fluorescence intensity. Interpretation of the deconvoluted histograms allowed the determination of the number of generations, the percentage of cells in each generation, and the fraction of non-proliferating cells at each observed time point. The non-proliferative fraction indicated the number of cells that had not undergone cell division, and, hence, remained in the parent generation (G.P.). The proliferation index (P.I.) was a measurement of the proliferative activity in the culture and represents the total expansion of the original population (cells seeded at Day 0); it represented the ratio of the total number of cells to the calculated number of cells in the parent generation. The total number of cells was determined by the summation of the Gaussian areas in each generation (A_k). To prevent the characterization of "false" generations due to background noise the proliferation index at each time point was calculated using a more stringent analysis to that outlined for the Cell Census Plus system: only those peaks with an area equal or greater than 10% of the average of the other generation peaks at that time point were designated as generation peaks. The original number of cells was calculated by the summation of the Gaussian areas divided by 2_k, where k equals the generation number. Hence, P.I. can be described as follows:

$$\text{P.I.} = \frac{\sum_{k=0}^{n} A_k}{\sum_{k=0}^{n} \frac{A_k}{2^k}}$$

5. DNA FRAGMENTATION ANALYSIS

Microscopic examination of PKM cultures in the *senescence* phase of development indicated the presence of several cells in the late stages of apoptotic cell death. To further examine this possibility several PKM cultures were established and the genomic DNA of representative cultures at the different phases of development was examined using the DNA fragmentation assay (12). Briefly, representative cultures in the proliferative, early senescence and late senescence phases of PKM development (3 cultures per group) were collected. Cells (about 5×10^6 per sample) were washed in serum free medium at 150xg for 10 min at 4°C, flash frozen in dry ice and 95% EtOH, and stored at –80°C until use.

Samples were lysed using a Proteinase K solution (20 mg ml^{-1}) for 10 minutes at 70°C. Genomic DNA was precipitated using ethanol (96-100%) and isolated using a silica gel membrane column (Qiagen) following the manufacturer's specifications. The DNA was then eluted from the silica gel membrane and stored at 4°C in sterile deionized water.

The genomic DNA was quantitated using the ethidium bromide agarose plate assay (13). Fragmented DNA was detected by agarose gel electrophoresis (0.8% gel; 25 V for 16 hours) followed by ethidium bromide staining (1 μg ml^{-1}; Gibco BRL). DNA was then visualized by ultraviolet examination. The size of the DNA was compared to that of standard molecular weight markers (100 bp DNA ladder; Boehringer Mannheim).

6. MOLECULAR ANALYSES

6.1. Construction of PKM phase-specific cDNA libraries

Two cDNA libraries were constructed for PKM at specific phases of culture development: a *proliferative phase* cDNA library and a *senescence phase* cDNA library. PKM in each of these phases of development were isolated, flash frozen, and stored at -70°C until used. mRNA was isolated using Trizol™ reagent (Gibco BRL) and the Oligotex mRNA isolation kit (Qiagen) according to the manufacturer's specifications: 2 µg of poly A^+ RNA were isolated for each of the two groups. The phage libraries were constructed by directional ligation of PKM cDNA into lambda ZAP bacteriophage using a cDNA synthesis kit, ZAP cDNA synthesis kit, and the ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). Non-amplified libraries were prepared as they provide a non-biased representation of the mRNA population at each of these phases of PKM culture development.

6.2. Identification of the goldfish CSF-1 receptor

6.2.1. cDNA library screening and isolation of CSF-1R transcripts

Alignment of human (X03663), mouse (X06368), cat (J03149), and *D. rerio* (AF240639) CSF-1R gene (c-*fms*) sequences led to the identification of conserved gene regions and subsequent construction of degenerate primers. CSF-1R gene primers were then used in the generation of goldfish-specific c-*fms* fragments.

PCR amplification of each c-*fms* fragment was performed as follows: 7 µl of goldfish genomic DNA template was added to 76 µl ddH_2O, dNTPs (0.2 µl of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10x PCR buffer (10 µl of 100mM Tris-HCl pH8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), forward and reverse primers (2.4 µl of each 20 µM solution; Invitrogen), and Taq DNA polymerase (1µl of 5 U/µl solution). PCR amplification was conducted in an Eppendorf Mastercycler Gradient™ thermal cycler. The amplification program consisted of a 5 min hot-start at 94°C, followed by 35 cycles of 95°C for 30 sec, 51°C for 30 sec, 72°C for 1.5 min, and a final elongation step of 72°C for 7 min. Amplified products were electrophoresed in an agarose gel, stained using ethidium bromide, and photographed on a UV transluminator. Each fragment was sequenced for confirmation of *fms* identity; in all, fragments directed against exons 4, 12, 14, 18, and 19 were isolated. These fragments were mixed in equimolar concentrations and subsequently utilized in the construction of a triple-labeled (α-^{32}P-dATP, α-^{32}P-dCTP, α-^{32}P-dGTP) mixed cDNA probe; the probe was labeled using the Random Primers DNA Labeling System (Invitrogen) and radiolabeled cDNA was purified using G-50 Sephadex Quick-Spin columns (Roche). The following list summarizes the primers used in the generation of goldfish-specific c-*fms* fragments:

Exon 4: Forward (5'-tgtgcggwwngaaggtsarga-3'); Reverse (5'-acsttgcgratvakgamrcc-3')
Exon 12: Forward (5'-agrtscgctggaaratcatc-3'); Reverse (5'-raactcccayytctcrttgta-3')
Exon 14: Forward (5'-atgarmrbgaggcyctsatgtc-3'); Reverse (5'-ccagvaggttgackatgttct-3')
Exon 18: Forward (5'-tgcatccaccgrgacrtr-3'); Reverse (5'-gttggagtcrttgatgatgtc-3')
Exon 19: Forward (5'-gcbcgyctsccwgtgaagtg-3'); Reverse (5'-gaaratctcccasagsadga-3')

The cDNA library screening procedure was performed as previously described (14). The unamplified PKM *proliferative phase* cDNA library was plated on NZY agar plates and plaque lifts were performed using nylon membranes (NEN Research). Membranes were prehybridized at 60°C in Hybrizol II (Oncor) prior to the addition of the mixed cDNA c-*fms*-specific probe and subsequently hybridized overnight. Following washing under stringent conditions (final wash with 0.1x SSC/0.1% SDS for 10 minutes at 65°C) the membranes were exposed to Kodak X-OMAT film and stored at -70°C for 1.5 to 72 hours. Autoradiographs were analyzed and putative positive plaques were matched to the original plaques on the NZY master plates. Isolated plaques were stored at 4°C in 500 μl of SM buffer + chloroform, and represented phage stocks of potential c-*fms* cloned transcripts.

6.2.2. PCR amplification of primary clones and secondary screening

Primary screen of 26,000 clones from PKM *proliferative* and *senescence* phase cDNA libraries yielded eight putative c-*fms* primary clones that were isolated and analyzed using a PCR-based secondary screen. The PCR amplification of each of these clones ensured the reliability of the secondary screen results. PCR amplification of each of these clones was performed as follows: 7 μl of each phage stock was added to 81 μl ddH$_2$O, dNTPs (0.2 μl of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10x PCR buffer (10 µl of 100 mM Tris-HCl pH8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), T3 primer (0.12 µl of 400 µM solution; 5'-attaaccctcactaaaggga-3'; Bioserve), T7 primer (0.12 µl of 400 µM solution; 5'-taatacgactcactataggg-3'; Bioserve), and Taq DNA polymerase (1 µl of 5 U/µl solution). PCR amplification was conducted in an Eppendorf Mastercycler Gradient™ thermal cycler. The amplification program consisted of a 3 min hot-start at 94°C, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 3.5 min, and a final elongation step of 72°C for 7 min. Amplified products were electrophoresed in an agarose gel, stained using ethidium bromide, and photographed on a UV transluminator.

For secondary screening, 1 µl of each PCR-amplified product corresponding to each of the putative fms primary clones was arrayed onto a gridded nylon membrane (NEN Research). The membrane was allowed to hybridize with the [^{32}P]-labeled fms-specific mixed cDNA probe. Pre-hybridization, hybridization, wash conditions, and processing of the secondary screen blots were as outlined above for the primary screen.

6.2.3. Tertiary screen

Clones confirmed to specifically bind to the fms mixed cDNA probe were re-plated on NZY agar plates and individual subclones were isolated. These were PCR-amplified as described above, confirmed to encode for a single-sized insert, and individually arrayed onto a gridded nylon membrane (NEN Research). Pre-hybridization, hybridization, wash conditions, and processing of the tertiary screen blots were as outlined above for the primary screen.

6.2.4. DNA sequencing and sequence analysis

The PCR amplified clone inserts corresponding to each of the confirmed fms positive clones were prepared for DNA sequencing using the QIAquick PCR purification kit (Qiagen). Purified inserts were sequenced using a DYEnamic™ ET terminator cycle sequencing kit (Amersham Pharmacia) and subsequent sequence analysis was performed at the Molecular Biology Service Unit (MBSU; Department of Biological Sciences, University of Alberta) using a PE Applied Biosystems 377 automated sequencer. Single-pass sequences were analyzed using Genetool™ (Biotools) and subsequent gene annonations were conducted using BLASTN and BLASTX for nucleotide and amino acid similarity comparisons, respectively (http://www.ncbi.nlm.nih.gov/BLAST/). Clones confirmed to encode for c-*fms* transcripts were fully sequenced and analyzed. The predicted protein sequence for each open reading frame (ORF) was compared to the GenBank non-redundant database using PSI-BLAST. Conserved motifs were identified manually or with ScanProsite (15), and subsequent predictions were based or a variety of analytical tools provided in the ExPASy World Wide Web proteomics server (http://www.expasy.org) (16). Sequence aligments were performed using CLUSTAL X Version 1.81 (17).

6.2.5. Recombinant sCSF-1R eukaryotic expression

6.2.5.1. Design of expression constructs, PCR amplification, and cloning

The novel soluble form of the CSF-1R (sCSF-1R) was expressed using an insect-based protein expression system. Expression primers flanking the sCSF-1R ORF were generated and the design of the protein expression construct was confirmed using the Gene Construction Kit 2 software package (SciQuest). PCR amplification of the protein expression construct insert was performed as follows: 7 μl of sCSF-1R clone template was added to 76 μl ddH$_2$O, dNTPs (0.2 μl of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10x PCR buffer (10 μl of 100 mM Tris-HCl pH8.3, 500 mM KCl, 15 mM MgCl$_2$, 0.01% (w/v) gelatin), forward primer (2.4 μl of 20 μM solution; 5'-gctatggccttt gctctcctgttcgtctgt-3' Invitrogen), reverse primer (2.4 μl of each 20 μM solution; 5'-atgaaa ctcacgctgaatgacg-3'; Invitrogen), and a15:1 ratio of *Pfu*:*Taq* DNA polymerases (1 μl of 5 U/μl solution). PCR amplification was conducted in an Eppendorf Mastercycler Gradient™ thermal cycler. The amplification program consisted of a 3.5 min hot-start at 94°C, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min, and a final elongation step of 72°C for 7 min. Amplification was confirmed by agarose gel electrophoresis of PCR products, staining using ethidium bromide, and observation on a UV transluminator.

The PCR-amplified sCSF-1R insert was cloned into the pIB/V5-His-TOPO® expression vector (Invitrogen) and transformed into chemically competent TOP10 *E. coli* (Invitrogen) according to the manufacturer's specifications. Once positive clones were isolated, restriction digests followed by gel electrophoresis verified the presence of insert and vector DNA. Plasmids were then sequenced as described above in order to confirm that inserts were ligated into the expression vector in the proper open reading frame (ORF). The following vector-specific primers were used in the sequencing reactions: Forward (5'-cgcaacgatctggtaaacac-3'), Reverse (5'-gacaatacaaactaagatttagtcag-3') (Invitrogen). Sequence data was analyzed using Genetool (Biotools) and Gene Construction Kit 2 (SciQuest) software packages.

6.2.5.2. Production of recombinant sCSF-1R and selection of stable cell lines

Drosophila KC embryonic cells (18) were grown in ESF-921 medium (Expression Systems) at 27°C. KC cells were subsequently transferred into Grace's insect medium (Sigma) and allowed to adhere. Liposome-mediated transfections were carried out by adding a mixture of 5 μg sCSF-1R expression plasmid, Grace's medium, and Cellfectin® Reagent (Invitrogen), followed by periodic mixing during a 4 hr incubation period. After 4 days cells were transferred to 25 cm^2 flaks and incubated in ESF-921 medium containing 75 μg/ml Blasticidin (Invitrogen) in order to select for stable transfectants. After two rounds of selection the stable cell lines were maintained in ESF-921 medium containing 10 μg/ml Blasticidin for 5-6 days at 27°C prior to passaging and collection of supernatants containing recombinant soluble CSF-1R.

6.2.5.3. Purification of recombinant sCSF-1R

Recombinant sCSF-1R was engineered to contain a C-terminal 6xHis tag and V5 epitope to facilitate subsequent purification and detection protocols. KC/sCSF-1R cell line supernatants were dialyzed against Ni-NTA wash buffer and concentrated in preparation for purification. Recombinant protein was purified using Ni-NTA agarose columns (Qiagen) according to the manufacturer's specifications (matrix binding capacity 5-10 mg/ml). Purified proteins were eluted in Ni-NTA wash buffer supplemented with 125 mM imidazole, dialyzed extensively against 1x D-PBS, and filter-steralized in preparation for immunodetection and analysis of biological activity. Total protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce) according to the manufacturer's protocols.

6.2.4.5. *Production of an anti-goldfish sCSF-1 receptor polyclonal antibody*

Purified recombinant sCSF-1R fractions (fractions 4 and 5) were combined and used as a source of antigen for the immunization of rabbits. Primary injection of the rabbit was performed by combining 700 μl of purified sCSF-1R (dialyzed and filter-sterilized) with 700 μl of Freund's Complete Adjuvant. Booster injections were done exactly as the primary immunizations but substituted with Freund's Incomplete Adjuvant. Specificity of the antibody was determined by reactivity with purified carp serum using Western blot analysis (Fig. 6.14).

6.2.5.5. *Immunodetection of purified recombinant sCSF-1R*

Purified recombinant sCSF-1R was detected using Western blot analysis by virtue of its C-terminal V5 epitope (refer to section 7.2 for details). Briefly, recombinant proteins were separated by SDS-PAGE under reducing conditions using a 12.5% polyacrylamide gel. Following SDS-PAGE, proteins were transferred to 0.2 μm nitrocellulose membranes (BioRad) and incubated O/N at 4°C in the presence of anti-V5 monoclonal antibody (mAb) (1:5000; Invitrogen). Membranes were washed, incubated with an HRP-conjugated mAb, and developed using the ECL Advance™ Western Blotting Detection Kit (Amersham Biosciences) according to the manufacturer's specifications.

6.2.6. *Biological activity of recombinant sCSF-1R*

6.2.6.1. *Flow cytometric characterization*

Two parameters were considered when analyzing the biological activity of sCSF-1R using flow cytometry. The first was based on changes in the rate of PKM migration through the various phases of development (*lag*, *proliferative*, and *senescence*); discriminating parameters are described in detail in Chapter V. In addition, PKH26 analysis determined the effects of recombinant sCSF-1R on PKM proliferation; the analysis was performed as described in section 6.3.

6.2.6.2. Colorimetric BrdU-based cell proliferation assay

The effects of recombinant sCSF-1R on PKM proliferation were also assessed using the Cell Proliferation ELISA BrdU colorimetric assay (Roche). This technique is based on the incorporation of the pyrimidine analog, 5-bromo-2'-deoxyuridine (BrdU), into the DNA of proliferating cells. Primary kidney macrophages, grown in the presence of PKM CCM (section 1.7), were isolated and seeded at a density of $2x10^4$ cells $well^{-1}$ in 96-well culture plates (Falcon). Cells were seeded in 75 μl of complete culture medium, treated with 25 μl of cell-conditioned supernatants with or without recombinant sCSF-1R, and incubated for 48 hours at 20°C. PKM were labeled with BrdU at 15 μM (final concentration) and incubated for 18 hours at 20°C. Culture plates were centrifuged at 300xg for 10 minutes, the supernatants were carefully removed by vacuum, and plates were incubated at 60°C for 1 hour. Cellular DNA was fixed in FixDenat solution, and the standard ELISA procedure was performed according to the manufacturer's protocols, by adding an anti-BrdU monoclonal antibody conjugated with peroxidase. Following washing, the color reaction was developed by adding a tetramethylbenzidine (TMB) substrate solution, and optical densities were determined at 450 nm-640 nm using a microplate spectrophotometer (Biotek). The colorimetric reaction was directly proportional to the number of proliferating PKM in culture (Fig. 2.3).

6.3. Identification of differentially expressed genes in the *proliferative* and *senescence* phases of PKM development

6.3.1. Differential cross-screening of PKM proliferative phase cDNA library

The differential cross-screening procedure was performed as previously described (14). The unamplified PKM *proliferative phase* cDNA library was plated on NZY agar plates and plaque lifts were performed using nylon membranes (NEN Research). For each plate duplicate lifts were made. Differential cross-screening of the library was performed using a [^{32}P] *proliferative phase* cDNA population as a probe to screen the first set of lifts and a [^{32}P] *senescence phase* cDNA population as a control probe to screen the second set of lifts.

Mixed cDNA probes from both phases of PKM growth were prepared separately. Briefly, polyA$^+$ RNA from *proliferative* and *senescence phase* preparations was purified independently as described above. Double-stranded cDNA was synthesized from 1 µg of poly A$^+$ RNA using the Timesaver cDNA synthesis kit (Amersham Pharmacia) and used unamplified to ensure the most accurate representation of mRNA populations in each of these phases. For the primary and secondary differential screens total cDNA populations were triple-labeled (α-^{32}P-dATP, α-^{32}P-dCTP, α-^{32}P-dGTP) using Prime-It II Random Primer Labeling kit (Stratagene) and radiolabeled cDNA was purified using STE Select-D G-50 spin columns (5Prime- 3Prime).

Membranes were prehybridized at 65°C in Hybrizol II (Oncor) for at least 3 hours prior to the addition of denatured probe and subsequently hybridized overnight at 65°C in a hybridization oven. Following washing under stringent conditions (final wash with 0.1x SSC/0.1% SDS for 10 minutes at 65°C) Kodak X-OMAT film was exposed to the membranes and stored at -70°C for 1.5 to 36 hours. Autoradiographs from the *proliferative phase* cDNA-probed membranes were compared to the *senescence phase* cDNA-probed membranes. Signals that showed different levels of intensity between parallel plaques were matched to the original plaques on the NZY master plates. Isolated plaques were stored at 4°C in 500 µl of SM buffer + chloroform, and represent phage stocks of cloned transcripts that are differentially expressed between the *proliferative* and *senescence phases*.

6.3.2. PCR amplification of primary clones and secondary screening

Primary screen of 9200 clones yielded 734 putative primary clones that were isolated and analyzed using a PCR-based secondary screen. The PCR amplification of each of these clones ensured the reliability of the secondary screen results. PCR amplification of each of these 734 clones was performed as follows: 7 µl of each phage stock was added to 81 µl ddH$_2$O, dNTPs (0.2 µl of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10x PCR buffer (10 µl of 100 mM Tris-HCl pH8.3, 500 mM KCl, 15 mM MgCl$_2$, 0.01% (w/v) gelatin), T3 primer (0.12 µl of 400 µM solution; 5'-attaaccctcacta aaggga-3'; Bioserve), T7 primer (0.12 µl of 400 µM solution; 5'-taatacga ctcactataggg-3'; Bioserve), and Taq DNA polymerase (1 µl of 5 U/µl solution). PCR amplification was conducted in an Eppendorf Mastercycler Gradient™ thermal cycler. The amplification program consisted of a 3 min hot-start at 94°C, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 3.5 min, and a final elongation step of 72°C for 7 min. Amplified products were electrophoresed in an agarose gel, stained using ethidium bromide, and photographed on a UV transluminator.

For secondary screening, 0.3µl of each PCR-amplified product corresponding to each of the 734 primary differential clones (i.e. putative differentially expressed gene identified following primary screen) was arrayed onto two gridded nylon membranes (NEN Research). Each membrane was allowed to hybridize with either a [^{32}P]-labeled *proliferative phase* mixed cDNA probe or a [^{32}P]-labeled *senescence phase* mixed cDNA probe. Pre-hybridization, hybridization, wash conditions, and processing of the secondary screen blots were as outlined above for the primary screen. The differential expression of 306 clones was confirmed, and the relative level of differential expression was used in the selection of clones for DNA sequencing.

6.3.3. DNA sequencing and sequence analysis

The PCR amplified clone inserts corresponding to each of the confirmed 306 differentially expressed clones were prepared for DNA sequencing using either the QIAquick PCR purification kit or QIAquick Gel extraction kit (Qiagen). Purified inserts were sequenced using a DYEnamic™ ET terminator cycle sequencing kit (Amersham Pharmacia) and subsequent sequence analysis was performed at the Molecular Biology Service Unit (MBSU; Department of Biological Sciences, University of Alberta) using a PE Applied Biosystems 377 automated sequencer. Single-pass sequences were analyzed using Genetool™ (Biotools) and subsequent gene annonations were conducted using BLASTX for amino acid similarity comparisons (http://www.ncbi.nlm.nih.gov/BLAST/).

6.3.4. Northern blot analysis

Three to ten micrograms of total RNA were subjected to electrophoresis on a 1% agarose formaldehyde gel and transferred overnight to Genescreen Plus nylon membranes (NEN Research) using standard protocols (13). Blots were screened with the corresponding single-labeled (α-^{32}P-dCTP) under stringent conditions (as described for the cDNA library differential screens) and subsequently exposed to Kodak X-OMAT film and stored at -70°C for 30 minutes to 72 hours.

The intensity of the bands in each of the autoradiographs was quantitated using Adobe™ Photoshop software package. In order to normalize the band intensities to possible variations in RNA loading, transcript bands were compared to the corresponding 18S RNA band intensities. Normalized values represent the intensity of the transcript band on the autoradiograph divided by the intensity of the 18S RNA band in the photograph of the corresponding sample in the original agarose formaldehyde gel photograph (19).

6.3.5. Macroarray analysis of putative macrophage differentiation markers

For generation of mixed cDNA probes, primary kidney macrophages (PKM) were incubated for 5-10 days at 20°C and isolated upon reaching the early, mid, and late *proliferative*, and early *senescence phases* of macrophage development. Pooled PKM were sorted under sterile conditions using a FACS Calibur flow cytometer equipped with a cell sorter (Becton Dickinson) as previously described (20). Sorted cells from either early progenitor, monocyte, or mature macrophage PKM subpopulations were collected in a 50 ml tube containing 10ml of medium supplemented with serum and antibiotics. Cells were concentrated by centrifugation (200xg for 10 min) and cell pellets were immediately flash frozen, and stored at -70°C until used. Total RNA for each of the three groups was isolated using Trizol™ reagent (Gibco BRL) and double-stranded cDNA generated using the SMART™ PCR cDNA synthesis kit (Clontech) according to the manufacturer's specifications. Mixed cDNA probes corresponding to each macrophage subpopulation were generated as described above for the primary and secondary differential cross-screens.

For macroarray construction, isolated clones corresponding to putative macrophage differentiation markers were PCR amplified as described above for the differential screen. The PCR amplified clone inserts were prepared for arraying using either the QIAquick PCR purification kit or QIAquick Gel extraction kit (Qiagen). 0.5 µl of each PCR-amplified product was arrayed in triplicate onto three gridded nylon membranes (NEN Research). Each membrane was allowed to hybridize with either a [^{32}P]-labeled early progenitor mixed cDNA probe, [^{32}P]-labeled monocyte mixed cDNA probe, or a [^{32}P]-labeled "mature" macrophage mixed cDNA probe. Pre-hybridization,

hybridization, wash conditions, and processing of the arrayed membranes were as outlined above for the primary differential screen. The level of differential gene expression between the three stages of macrophage differentiation was quantitated by comparing the relative signal intensities corresponding to each selected gene between each of the three resulting autoradiographs. The intensity of the spots in each of the autoradiographs was quantitated using Adobe™ Photoshop software package. In order to normalize the spot intensities to possible variations in cDNA loading, triplicate transcript spots were compared to the corresponding beta-actin spot intensities. Validation of beta-actin as an appropriate control in PKM gene expression analysis can be observed in Figure 7.4. Normalized values represent the average intensity of the transcript spots (n=3) divided by the average intensity of the beta-actin spots on the autoradiograph.

6.3.6. Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) analysis

Cells corresponding to the early hematopoietic progenitor, monocyte, and mature macrophage groups were isolated under sterile conditions using a FACScalibur flow cytometer equipped with a cell sorter (Becton Dickinson) as described above. These cells were used to compare the differential levels of gene expression for selected transcripts across the three stages of macrophage differentiation previously identified in PKM cultures (20). This procedure was useful for the analysis of transcripts resulting from mRNA processing of pro-mRNA species; therefore, it overcomes the cross-hybridization of gene-specific cDNA probes with both species during the macroarray analysis procedure. A comparison between the results of both techniques is presented in Figure 7.7 (Chapter 7) as validation of the RT-PCR protocol for analysis of PKM gene expression.

Total RNA was isolated using Trizol™ reagent (Gibco BRL) and double-stranded cDNA generated using the SMART™ PCR cDNA synthesis kit (Clontech) according to the manufacturer's specifications. Semi-quantitative PCR amplification of each of the selected transcripts was performed as follows: 1 µl of the appropriate double-stranded cDNA template was added to 81.6 µl ddH$_2$O, dNTPs (1.6 µl of a dATP, dCTP, dGTP, dTTP 25 mM solution), 10x PCR buffer (10 µl of 200 mM Tris-HCl pH8.3, 500 mM KCl, 50 mM MgCl$_2$, 0.5% (w/v) gelatin), forward and reverse primers (2.4 µl of each 20 μM solution), and a 100:1 Taq:PFU DNA polymerase blend (1 μl of 5 U/μl solution). PCR amplification was conducted in an Eppendorf Mastercycler Gradient™ thermal cycler. The amplification program consisted of a 1 min hot-start at 95°C, followed by 18-39 cycles of 95°C for 10 sec, 50-55°C for 10 sec, 72°C for 3 min, and a final elongation step of 72°C for 7 min. Amplified products were electrophoresed in an agarose gel, stained using ethidium bromide, and photographed on a UV transluminator. The identity of each product amplified with each primer pair was confirmed by DNA sequencing and subsequent analysis using Genetool™ (Biotools) and BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/), as described above. The following list summarizes the primers used in this study:

β-actin: Forward (5'-tcgctgccctggtcgttgata-3'); Reverse (5'-ggcggcggttcccatctc-3')
Legumain: Forward (5'-tgtcgttgcctgtgattactgaa-3'); Reverse (5'-cctggtatcctccctgacaca-3')
CD63: Forward (5'-cgacacctcagccatcatca-3'); Reverse (5'-tggcacagagatatacaggatga-3')
Granulin: Forward (5'-cgtgctgtcgttctccttttggta-3'); Reverse (5'-actcagtctcgctcttccattta-3')
C/EBPβ: Forward (5'-agccatcgacttcagcatctatttg-3'); Reverse (5'-gcgcattttggctttgtctct-3')
c-Kit: Forward (5'-gggatgtccaaagcasacactg-3'); Reverse (5'- csggctagtccaaartcacaga-3')
AC133: Forward (5'-tggtctcctggatcttcatgtta-3'); Reverse (5'-gcttccccgtgcagttcatct-3')
PU.1: Forward (5'-tgccggagagccattttacag-3'); Reverse (5'-cccgtctttccgtagtttctcag-3')

7. PROTEIN ANALYSIS

7.1. SDS-PAGE electrophoresis

Proteins of interest were analyzed by SDS-PAGE using methods previously described (21). Total protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce) according to the manufacturer's protocols. Samples were dissolved in an equal volume of 2X sample buffer (62.5 mM Tris-HCL pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue, 10% 2-β-mercaptoethanol; Sigma), heated at 95°C for 5 minutes, and electrophoresed through 3% stacking, 7.5 - 15% separating discontinuous polyacrylamide gels. Electrophoresis was carried out in 1x SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3; Sigma) at 10 mA per gel through the stacking gel, followed by 20 mA per gel through the separating gel. Protein bands were visualized by staining the gels with 0.04% Coomassie Brilliant

Blue R-250 (BioRad) dissolved in 40% methanol/10% acetic acid for 30 min followed by destaining with 40% methanol/10% acetic acid. Alternatively, protein bands were visualized by staining the gels with a silver staining kit according to the manufacturer's protocols (BioRad).

7.2. Western blot analysis

Following protein separation by SDS-PAGE as indicated above, the gels were equilibrated in transfer buffer (25 mM Tris-HCL, 192 mM Glycine, 20% (v/v) methanol, pH 8.3) for 5 min. Protein samples were transferred onto 0.2 µm nitrocellulose membrane (BioRad) at 100 V for 1 hour. After protein transfer, the nitrocellulose was reversible stained with Ponceau S (Sigma) for visualization of the SDS-PAGE molecular weight standards (BioRad). The standards were removed by cutting with a scalpel and the remaining nitrocellulose was washed with milli-Q water to remove excess stain. Nitrocellulose membranes were then placed in blocking buffer (100 mM Tris-HCL, 0.9% (w/v) NaCl, 0.5% (w/v) BSA, and 0.1% Tween 20, pH 7.5) and incubated for 30 min at room temperature. Following addition of primary antibody (eg. polyclonal anti-sCSF-1R), the membrane was incubated overnight on a rocking platform at 4°C and developed using the ECL Advance™ Western blotting detection kit (Amersham Biosciences) according to the manufacturer's specifications.

8. STATISTICS

One and two-way analysis of variance using SuperAnova software (Abacus) for the Power Macintosh was used for determining significance between control and experimental groups. Probability level of $P < 0.05$ was considered significant.

Table 2.1. Composition of medium used to culture goldfish macrophages and leukocytes.[a]

Reagent or Solution	Amount	Special requirements & supplier
Milli-Q®-water	700 ml	endotoxin free, 18MΩ
HEPES	7.00 g	(Sigma)
KH_2PO_4	0.688 g	(BDH)
K_2HPO_4	0.570 g	(BDH)
NaOH	0.75 g	(Fisher)
$NaHCO_3$	0.34 g	(BDH)
Hank's balanced salt solution	80 ml	10X solution, no Mg^{2+} or Ca^{2+}
MEM amino acid solution	25 ml	50X (Gibco)
MEM non-essential amino acids	25 ml	100X (Gibco)
sodium pyruvate	25 ml	100X (Gibco)
MEM vitamin solution	20 ml	100X (Gibco)
nucleic acid solution	20 ml	solution containing 2.5 mM each of adenosine, cytidine, hypoxanthine, thymidine, and uridine (Sigma)
L-glutamine	0.584 g	(Sigma)
2-b-mercaptoethanol	0.007 ml	(Sigma)
insulin	0.010 g	(Sigma)
GFL-15 medium	1000 ml	Base medium comprised of equal volumes of DMEM (high glucose, no pyruvate, no sodium bicarbonate, Gibco) and Leibovitz's medium (Gibco).

[a] Reagents were added in the order listed. Medium was balanced to pH 7.4 using 5 M NaOH, and the total volume brought up to 2 litres.

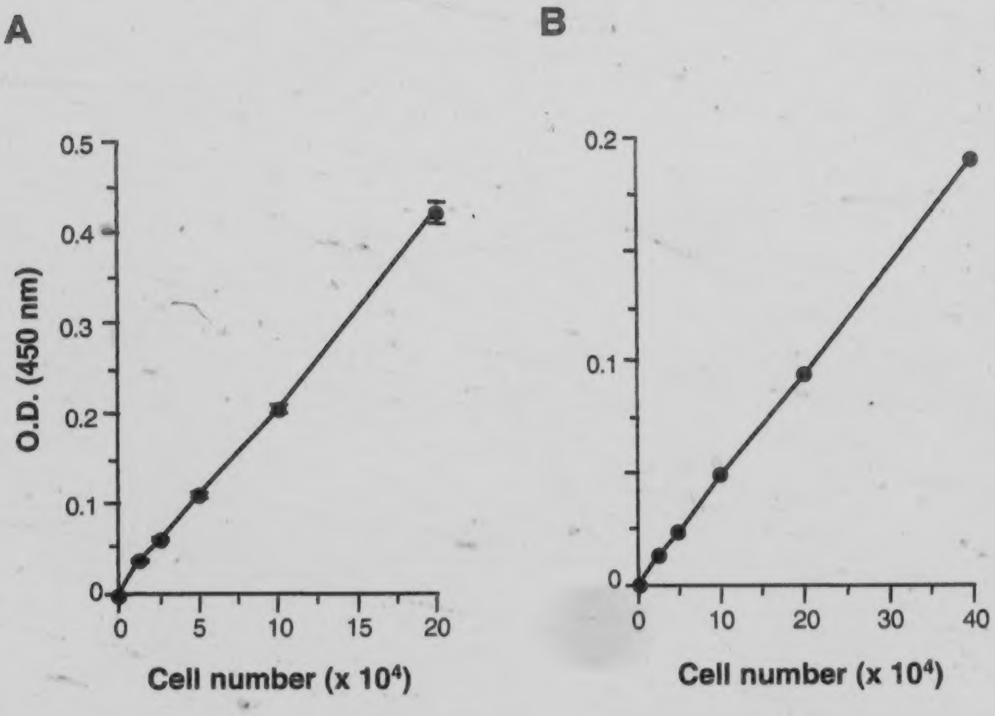

Figure 2.1: The reduction of the tetrazolium salt XTT to a soluble formazan in the presence of co-enzyme-Q by GMCL (A) and kidney leukocytes (B). The colorimetric reaction (O.D. 450 nm) was directly proportional to the number of viable cells within culture. Each point represented the mean ± SEM of quadruplicate cultures for GMCL and mean ± SEM of quadruplicate cultures obtained from kidney leukocytes of 4 fish.

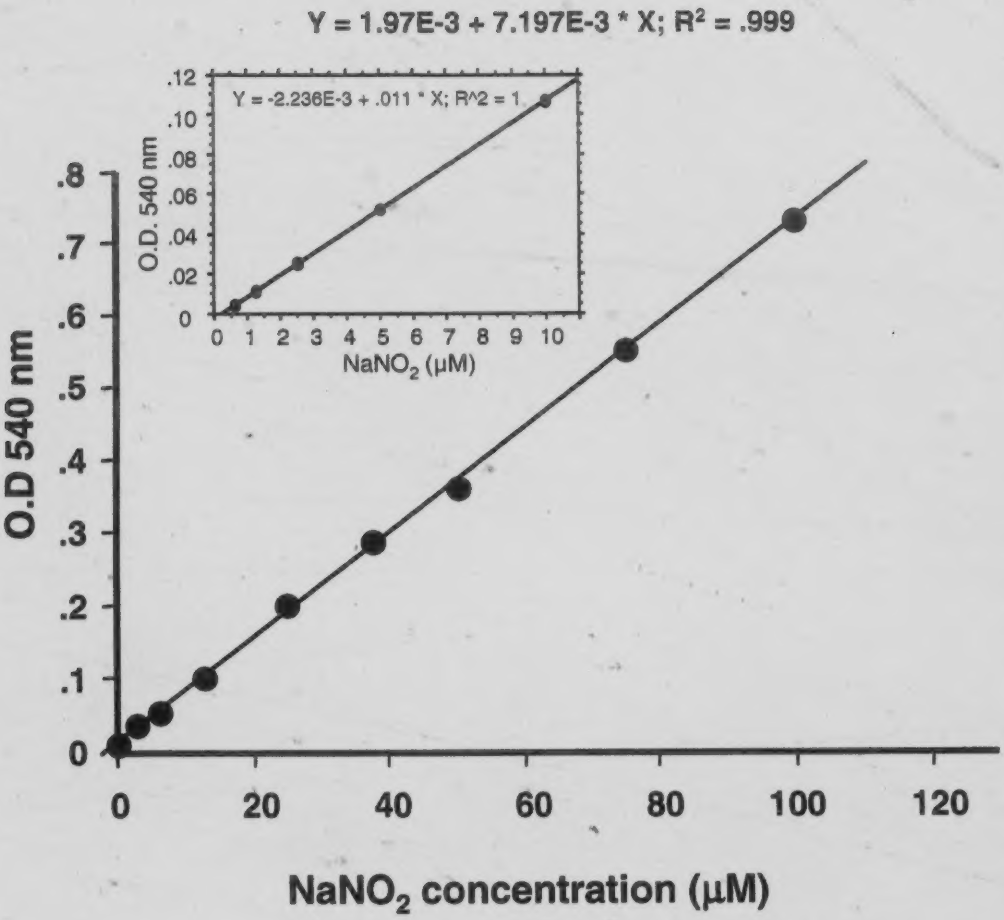

Figure 2.2: Sodium nitrite standard curve for the determination of NO production using the Griess reaction. Inset: Sodium nitrite standard curve for low values of nitrite (1.0-10 µM).

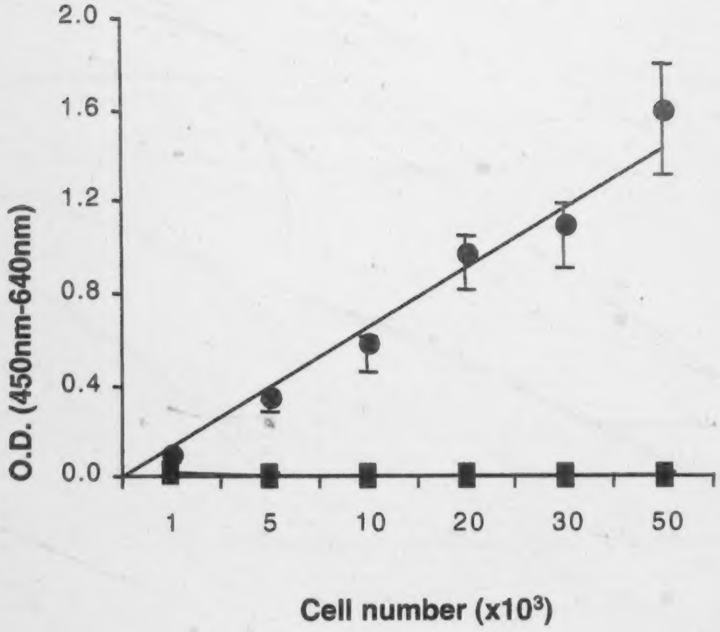

Figure 2.3: Measurement of primary kidney macrophage proliferation by BrdU incorporation. The colorimetric reaction (O.D. 450nm-640nm) upon addition of BrdU was directly proportional to the number of proliferating cells within culture (filled circles). In contrast, the background control (filled squares) indicated that no significant binding of the anti-BrdU antibody to the cells occurred in the absence of BrdU. Each point represents the mean ± SEM of triplicate cultures.

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CHAPTER III:

GENERATION OF MACROPHAGE CULTURES FROM GOLDFISH KIDNEY HEMATOPOIETIC TISSUES IN RESPONSE TO ENDOGENOUS MACROPHAGE GROWTH FACTOR(S)[2]

1. INTRODUCTION

Over the last 30 plus years colony stimulating factor (CSF) research has led to a significant improvement in the understanding of the mechanisms that control the generation of immune cells from early pluripotent precursors. Many of these breakthroughs have been a direct result of improvements in cell culture techniques from early colony-forming assays, which tested growth factor activity from cell and organ culture supernatants, to the more recent developments spearheaded by defined serum-free culture media, flow-through strategies, and systems where hematopoietic progenitor cells were co-cultured with defined feeder layer cell lines (1-3). Currently, CSFs are at the center of widespread therapeutic applications aimed at modulating immune responses against diseases.

Our laboratory is interested in the immune responses of fish to pathogens. The long-term goal is to establish a fish immune model system aimed at improving the innate host defense strategies against fish pathogens. To this end, we have chosen to study the macrophage, a central cell of innate and acquired immune responses, and have focused our efforts on the characterization of its development and function. In the early 1990s we established a goldfish macrophage cell line, GMCL, from kidney hematopoietic tissues that showed morphological, cytochemical, and functional similarities to mammalian macrophages (4). These cells were: 1) morphologically similar to mammalian macrophages as determined by light and transmission electron microscopy; 2) adherent to plastic and glass; 3) able to form multinucleated giant cells in culture; 4) non-specific esterase positive; 5) chemotactic; 6) phagocytic against sheep red blood cells and *Leishmania major*, an obligate intracellular pathogen of mammalian macrophages; and 7) efficient producers of reactive oxygen and nitrogen intermediates in response to stimulation with macrophage activating factors (MAF) and/or bacterial LPS.

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[2] Portions of this chapter have been published:
Neumann, Barreda, and Belosevic (1998) *Dev. Comp. Immunol.* 22(4): 417-432.
Neumann, Barreda, and Belosevic (2000) *Fish and Shellfish Immunol.* 10(1): 1-20.
D.R. Barreda was involved in the collection of data, analysis, and writing of the manuscripts.

In this chapter, I present results of studies whose aim was to establish primary macrophage cultures *in vitro* from goldfish hematopoietic tissues. I characterized the predominant cell in the cultures as the macrophage based on: a) non-specific esterase staining; b) morphological similarity to the spontaneously growing GMCL, maintained in culture for over 7 years; c) phagocytosis of the bacterium, *A. salmonicida*; and d) production of reactive oxygen and nitrogen intermediates in response to macrophage activating factors and/or LPS. I report that these *in vitro*-derived macrophages developed in response to endogenous macrophage growth factors (MGF) present in macrophage culture supernatants. Moreover, developing primary kidney macrophage (PKM) cultures, like GMCL, are comprised of three distinct subpopulations of cells based on flow cytometric analysis of their size and internal complexity parameters. Subsequent characterization of these three subpopulations based on morphological, cytochemical, and functional analyses suggested that they represented cells of the macrophage lineage temporally arrested at subsequent stages of macrophage differentiation.

2. RESULTS

2.1. Production of macrophage growth factors by a goldfish macrophage cell line

Primary goldfish macrophage cultures were established *in vitro* using leukocytes isolated from kidney hematopoietic tissues of the goldfish, *Carassius auratus*, as described in Chapter II. Kidney leukocytes were stimulated to develop in culture by adding cell conditioned medium (CCM) obtained from 7-10 day old goldfish macrophage cell line (GMCL) cultures.

Kidney leukocytes treated with GMCL-derived supernatants proliferated significantly more than control cell cultures suggesting the presence of soluble growth-promoting factor(s) in GMCL supernatants (Fig. 3.1). These differences in the proliferative responses were particularly marked when relatively few kidney leukocytes were used as the seeding inoculum (Fig. 3.1). Wells seeded with $6.25x10^3$, $1.25x10^4$, or $2.5x10^4$ cells and cultured in the presence of 25% (v/v) GMCL-derived MGF had significantly more cells after 8 days of cultivation than control kidney leukocyte cultures not supplemented with GMCL-conditioned supernatants ($P < 0.05$). In contrast, the presence of GMCL-derived MGF did not significantly alter cell number when kidney leukocytes were seeded at higher densities (greater than $5x10^4$, $P > 0.05$), suggesting that goldfish kidney leukocytes, seeded above a threshold density *in vitro*, also produced endogenous MGF in sufficient quantities for induction of cell growth. The proliferation-inducing activity of GMCL-derived CCM in kidney leukocyte cultures was also dose dependent; cell growth for kidney leukocytes treated with GMCL-conditioned supernatants was significantly greater than that of the control group (no CCM added) particularly at higher CCM concentrations (Fig. 3.2).

2.2. Endogenous production of macrophage growth factor(s) by goldfish kidney leukocytes

Analysis of cell growth using $5x10^4$ cells/well in the presence and absence of GMCL-derived supernatants provided the first evidence of endogenous macrophage growth factor production by goldfish kidney leukocytes (Fig. 3.1). This was supported by subsequent observations of the growth kinetics of kidney leukocytes seeded at two cell densities in the presence or absence of GMCL-derived CCM (Fig. 3.3). Cells in wells seeded with $6.25x10^3$ kidney leukocytes proliferated only upon addition of exogenous GMCL-derived CCM (Fig. 3.3). In contrast, cells in cultures where the initial cell density was $2.5x10^4$ kidney leukocytes, proliferated over the 16-day incubation period both in the presence or absence of exogenous GMCL-derived CCM. The $2.5x10^4$ kidney leukocyte cultures incubated in the presence of GMCL-conditioned supernatants, however, had significantly greater cell numbers than controls at 12 and 16 days of cultivation ($P < 0.05$ for both days), suggesting that the addition of exogenous growth factor(s) in the form of CCM contributed significantly to the induction of cell growth.

When $1x10^5$ kidney leukocytes were seeded into wells of a 96-well tissue culture plate, cells proliferated in the absence of GMCL-conditioned supernatants (Fig. 3.4A).

Moreover, the supernatants obtained from these newly established cultures significantly enhanced the proliferation of kidney leukocytes seeded at 1.25x10^4 cells/well, providing direct evidence for endogenous production of MGF (Fig. 3.4B). Growth-enhancing supernatants obtained from these kidney leukocyte cultures were termed kidney leukocyte-derived cell conditioned medium (KL-derived CCM).

2.3 Characterization of *in vitro*-derived goldfish primary kidney macrophages

2.3.1. Characterization of kidney leukocyte cultures

Goldfish kidney leukocytes cultured in the presence of GMCL or KL-derived supernatants resulted in cultures consisting of both non-adherent and adherent cell populations. The non-adherent population was used for characterization of the predominant cell types since this population comprised the vast majority of cells in the cultures (Fig. 3.5A). The adherent cell population displayed typical macrophage morphology, spreading extensively on tissue culture flasks and in many cases forming giant multinucleated cells (Fig. 3.5B).

Preliminary characterization of kidney leukocyte cultures indicated that the predominant cell-type that developed upon addition of GMCL or KL-derived CCM was the macrophage based on the following criteria: 1) non-specific esterase staining; 2) morphologic similarity to GMCL, previously established in our laboratory and maintained in culture for over 7 years; 3) phagocytosis of the bacterium *A. salmonicida*; and 4) production of reactive oxygen and nitrogen intermediates upon stimulation with macrophage activating factors (MAF) and/or bacterial lipopolysaccharide (LPS). However, significant heterogeneity was observed in the cytological appearance of cultured cells, as well as in their non-specific esterase staining and functional responsiveness to MAF and /or LPS.

2.3.2. Flow cytometric analysis

Analysis of the size and internal complexity parameters of primary kidney macrophages using flow cytometry indicated the presence of three distinct subpopulations after 8 days of cultivation (Fig. 3.6). All three subpopulations were present in both adherent and non-adherent cell culture fractions (Fig. 3.7); therefore, in order to minimize cell damage associated with removing adherent cells from plastic surfaces, we decided to utilize the more abundant non-adherent cell fraction in subsequent experiments. Interestingly, GMCL cultures displayed a similar flow cytometric profile to PKM (Fig. 3.8). The three subpopulations of cells were designated as R1, R2, and R3-type "macrophages". The relative proportions of each of these subpopulations found in fourteen PKM cultures were presented in Table 3.1.

In order to determine the kinetics for the appearance of each macrophage subpopulation, flow cytometric analysis was performed on twenty PKM cultures between Day 0 and Day 8 (Fig. 3.9). At Day 0 cultures were predominantly composed of R1-type macrophages. Subsequent cultivation resulted in a decrease in the proportion of R1-type macrophages and a progressive increase in R2- and R3-type macrophages. However, the appearance of R2- and R3-type macrophage subsets varied between cultures established using kidney leukocytes of different fish. In some cases, R2-type macrophages developed first, whereas in others their appearance in cultures occurred following that of R3-type macrophages; similarly, the proportions of cells on Day 8 cultures varied considerably, particularly in R2 and R3 subpopulations (Table 3.1).

The identification of the three distinct "macrophage" subpopulations by flow cytometry led us to re-examine the morphological, cytochemical, and functional characteristics of cells in PKM cultures. These results are described below.

2.3.3. Morphological and cytochemical analysis of primary kidney macrophage subpopulations

The three PKM subpopulations were sorted using a FACS Calibur flow cytometer equipped with a cell sorter (Becton Dickinson) based on the size and internal complexity parameters, as described in Chapter II. Subsequently, microscopical examination of cells using stained cytospin preparations allowed examination of their morphological characteristics. Each PKM subpopulation was also stained for non-specific esterase (using α-naphthyl acetate esterase), acid phosphatase, and myeloperoxidase to examine their cytochemical characteristics.

R1-type macrophages were small cells (~6-10 μm in diameter) with a high nucleus to cytoplasm ratio (Fig. 3.10). These cells stained positively for acid phosphatase, but did not stain for myeloperoxidase or non-specific esterase (Fig. 3.11).

R2-type macrophages were large irregularly shaped cells (~12-20 μm in diameter), with a low nucleus to cytoplasm ratio, and often had extensive vacuolization and membrane ruffling (Fig. 3.10). R2-type cells stained positively for acid phosphatase, and had localized staining for myeloperoxidase (Fig. 3.11). These cells exhibited variable staining for non-specific esterase (Fig. 3.11). The morphologic and cytochemical appearance of these cells was similar to that of mature mammalian tissue macrophages.

R3-type macrophages were large round cells (~12-15 μm in diameter) with a low nucleus to cytoplasm ratio, and often had eccentrically placed "kidney" shaped nuclei (Fig. 3.10). The morphologic appearance of these cells was similar to that of mammalian monocytes. Interestingly, a human monocyte-like cell line, U937, segregated to the same region as R3-type macrophages using the same flow cytometer settings for analyzing PKM (data not shown). R3-type macrophages were acid phosphatase positive, and showed strong cytoplasmic staining for myeloperoxidase (Fig. 3.11). Like R2-type macrophages, the R3-type macrophages showed variable staining for non-specific esterase (Fig. 3.11).

2.3.4. Functional characteristics of primary kidney macrophage subpopulations

Primary kidney macrophages were highly phagocytic, capable of ingesting large numbers of bacteria (Fig. 3.12). Moreover, both R2 and R3-type macrophages actively phagocytosed *Leishmania major*, with 17% of R3-type macrophages and 14% of R2-type macrophages becoming infected after 3 hr of incubation (10:1 parasite to macrophage ratio; data not shown). R1-type macrophages were not phagocytic, although parasites could often be seen adhering to the cell surface of R1-type macrophages.

The R2 and R3-type macrophages were tested for their ability to produce reactive nitrogen and oxygen intermediates in response to activation with macrophage activating factors (MAF) and/or LPS. Because R1-type macrophages constituted only a small proportion of the cells in cultures (less than 13% of the total population) and were significantly more susceptible to mechanical damage due to sorting, obtaining sufficient numbers of viable cells for functional studies was impractical.

R2-type macrophages activated with MAF and LPS appeared to be the cell type responsible for most of the nitric oxide production in PKM cultures. Interestingly, R2-type macrophages produced little or no nitric oxide when treated with MAF or LPS alone, whereas they produced copious amounts of nitric oxide when stimulated by both MAF and LPS (Fig. 3.13). In contrast, R3-type macrophages produced comparatively small amounts of nitric oxide when activated with MAF and LPS (Fig. 3.13).

Production of reactive oxygen intermediates was determined by priming the respiratory burst of R2 or R3-type macrophages with MAF and/or LPS and determining their respiratory burst response after 6 or 48 hrs. R2-type macrophages were primed for enhanced respiratory burst response after 6 hrs of stimulation with MAF and/or LPS, and they exhibited sustained respiratory burst activity after prolonged treatment with MAF and/or LPS (Fig. 3.14). In contrast, R3-type macrophages were primed for enhanced respiratory burst response after 6 hrs of stimulation with MAF and/or LPS, but subsequently underwent de-priming of their primed state (Fig. 3.14). Thus, R3-type macrophages stimulated with MAF and/or LPS for 48 hrs had a significantly lower respiratory burst response compared to their counterparts stimulated for only 6 hrs. This de-priming effect was most notable in R3-type macrophages stimulated with LPS only, where macrophages stimulated for 48 hr exhibited a respiratory burst response similar to that of unstimulated controls.

3. DISCUSSION

The renewal of macrophage populations is essential for the proper maintenance of immune competence and homeostasis. In mammalian systems this process is mediated by growth factors present in circulation or produced locally by cells of the surrounding microenvironment. Early stimulation of mammalian hematopoietic progenitors with GM-CSF, IL-3, SCF/c-kit ligand, and/or Flt-3 ligand result in the initiation of developmental events (1, 5-9). Subsequent stimulation with CSF-1 and IL-6 at later stages of differentiation guides the developing cells along the macrophage lineage (6, 10). Ultimately, integration of these cytokine-mediated developmental signals with others resulting from discrete extracellular and intracellular events leads to the generation of mature effector macrophages capable of fulfilling their immune defense and housekeeping roles.

The results described in this chapter indicate that fish macrophages display several characteristics similar to those of mammalian macrophages. However, there also appear to be marked differences between mammalian and fish macrophages. Notably, goldfish macrophages develop *in vitro* in response to endogenous macrophage growth factor(s) (MGF). In contrast, in tissue cultures prepared from hematopoietic organs, mammalian macrophages are unable to support their own growth, as they are not able to produce appreciable levels of autocrine factors. Instead, they are dependent on T cells, endothelial cells, and fibroblasts for the production of growth-inducing hematopoietic mediators (11-18).

Cell conditioned media from both a goldfish macrophage cell line (GMCL) as well as primary kidney macrophage (PKM) cultures support the growth of subsequent PKM cultures. Addition of these supernatants to newly seeded cultures facilitated their growth and reduced the lag time required for initiation of proliferation events. This phenomenon has also been observed by others. Ganassin and Bols (19) demonstrated that a macrophage-like cell line, established from rainbow trout, required supernatants from previous cultures for continuous passage. Moreover, they have demonstrated that this cell line actively proliferated in response to its own CCM. Our results and those of others (4, 19, 40-43) suggest that, unlike mammalian macrophages, teleost macrophages directly mediate self-propagation and renewal through the production of endogenous growth factor(s).

The kinetics of PKM growth in culture were very similar to those reported for mammalian macrophages derived from bone marrow cultures in the presence of conditioned medium from the L-929 fibroblast cell line (20). PKM cultures undergo a lag phase in growth during the first 4 days of culture. During this lag phase cell growth was minimal and non-proliferating or short-lived cells died within culture, as indicated by the extensive cell debris generally present after 2-4 days of cultivation. However, debris was rapidly cleared by the remaining cells, and over the next 4 days of cultivation a significant increase in the number of cells in culture occurred. At that stage, cells in the cultures were defined as macrophages based on the following criteria: (1) cytological appearance; (2) non-specific esterase activity; (3) phagocytic ability; and (4) production of reactive oxygen and nitrogen intermediates. Both adherent and non-adherent cells were present in these cultures: adherent cells spread extensively on tissue culture flasks and often formed multi-nucleated giant cells. The non-adherent cells grew in clusters and attained significant cell densities in culture (greater than 5 x 10^5 to 1 x 10^6 cells/mL).

Primary kidney macrophage cultures were composed of a heterogeneous population of cells, as determined by flow cytometry, morphology, cytochemistry, and physiological function. Three distinct macrophage sub-populations were observed in both PKM and GMCL cultures using flow cytometry, and appeared to represent macrophages temporally arrested at distinct differentiation junctures in development. The R1 subpopulation composes the majority of the cells in PKM cultures at Day 0 along with non-macrophage cells that appeared to die by about Day 4. At that time the heterogeneity associated with the R1 subset decreased significantly and yielded the subpopulation of early macrophage progenitor-like cells. Consistent with the decrease in cells of the R1 subset there was a progressive increase in cells within the R3 and R2 subpopulations. Morphologically, these cells resembled mammalian monocytes and "mature" macrophages, respectively. The progressive increase in monocyte-like cells as cultures developed presents an exciting possibility, whereby PKM cultures may provide an important tool for the characterization of the pathways governing fish macrophage differentiation. This was important because we now had the opportunity to examine the maturation process of fish macrophages in response to endogenous growth factors, and in the context of three continually interacting subsets, each at a distinct stage of fish macrophage differentiation.

At present, it is not known which macrophage sub-population(s) (i.e. R1, R2, or R3-type macrophages) was responsible for MGF production. It also remains unclear whether MGF: a) supported macrophage growth alone; b) acted in combination with additional growth factors that may have stimulated our "seeder" cells prior to the isolation from goldfish kidney hematopoietic tissues; or c) required physical associations between cells in the three identified PKM subpopulations. Resolution of these issues requires further characterization of each macrophage subpopulation identified in our cultures and the availability of recombinant MGF.

Significant heterogeneity also remained within cells in each of these subpopulations, which was particularly evident following analysis of their cytochemical characteristics. This variability may be associated with the cellular heterogeneity that was typical of the macrophage lineage (20-29). As such, significant heterogeneity has been observed in different mature mammalian macrophage populations even in the case of non-specific esterase staining, widely considered as a determinant marker of macrophages (24, 30). Alternatively, the variability within PKM subpopulations may point to discrete changes in the cellular phenotype that resulted from a continuum of differentiation-related events occurring as cells progressed through their developmental pathways. If this was the case, it is not surprising that such differences cannot be separated solely based on size (FSC-H) and internal complexity (SSC-H) flow cytometric parameters. Surely, the future availability of recombinant fish cytokines and an improved understanding of discrete gene and protein expression changes that occur as fish macrophages develop, will facilitate obtaining answers to these questions.

Analysis of macrophage activation following stimulation with MAF and/or LPS indicated that R2- and R3-type macrophages were functionally distinct. R2-type macrophages were potent producers of nitric oxide when stimulated with MAF + LPS. As such, R2-macrophages demonstrate the "classical" macrophage response: little or no production of nitric oxide when stimulated with MAF or LPS, but copious production when co-stimulated with both factors. In contrast, R3-type macrophages produced only small amounts of nitric oxide in response to stimulation with MAF or LPS alone, or a combination of these factors. This was similar to reports on nitric oxide responses of human monocytes (31, 32), suggesting that the acquisition of the capacity to generate nitric oxide may require differentiation of fish monocytes to mature macrophages. This was consistent with reports indicating that maturation of monocytes leads them to acquire the capacity to produce nitric oxide in response to stimulation with cytokines and LPS (33, 34). However, it should be noted that there are significant differences in the ability of mature macrophages from various species to produce nitric oxide (32, 35-37).

The R2- and R3-type macrophages also displayed distinct respiratory burst priming kinetics when stimulated with MAF and/or LPS. R2-type macrophages were primed for enhanced respiratory burst responses after only 6 hrs of treatment with MAF and/or LPS. Their ability to mount this response continued to increase the longer they were treated with MAF and/or LPS (i.e. 48 hrs). The R3 monocyte-like cells, in contrast, were primed for enhanced respiratory burst response after 6 hrs of stimulation with MAF and/or LPS, but subsequently underwent deactivation of their primed state. Thus, R3-type macrophages stimulated with MAF and/or LPS for 48 hrs had a significantly lower respiratory burst response compared to their counterparts stimulated for only 6 hrs. Interestingly, this rapid priming and subsequent deactivation of the respiratory burst response in R3-type macrophages was similar to the respiratory burst kinetics previously described in our laboratory using GMCL and PKM macrophages (38, 39). These data suggest that the mechanisms for induction of fish macrophage antimicrobial responses may be dependent upon the concerted actions of functionally distinct macrophage sub-populations, and warrant future examination of the role that interactions between PKM subpopulations may have on the modulation of fish macrophage antimicrobial responses.

There are also important practical aspects to the findings reported in this chapter. The isolation and cultivation procedures described in this chapter may be used to obtain relatively large numbers of highly purified functional macrophages of fish (>1 x 10^7/fish). Moreover, this technique may be used to establish long-term macrophage cultures. On four separate occasions, we have been able to propagate primary macrophage cultures for over two years, demonstrating the extremely high proliferative potential of primary macrophage cultures of the goldfish. In one experiment, kidney leukocytes seeded at 1x10^5 cells/well (and taken from only two wells) were propagated to numbers equivalent to fifteen 75 cm^2 tissue culture flasks after approximately 8 months in culture. Previously, progress in understanding the roles that macrophages play in mediating humoral and cellular immunity in teleosts has been hindered by the inability to obtain sufficient numbers of macrophages; as a result, this technique may prove indispensable to research efforts directed at understanding the fundamental role of macrophages play in teleost host defense as well as the mechanisms modulating the generation of mature effector macrophages from early hematopoietic progenitors.

Table 3.1: Proportion of R1, R2, and R3-type macrophages in day 8 primary kidney macrophage cultures[a]

Macrophage subpopulation	Percent of PKM culture (±SEM)	Range (%)
R1	13.1 ± 0.79	8.0 - 17.0
R2	29.4 ± 2.35	14.7 - 44.7
R3	34.1 ± 3.97	10.4 - 58.0

[a] Data from fourteen separate primary kidney macrophage (PKM) cultures analyzed by flow cytometry.

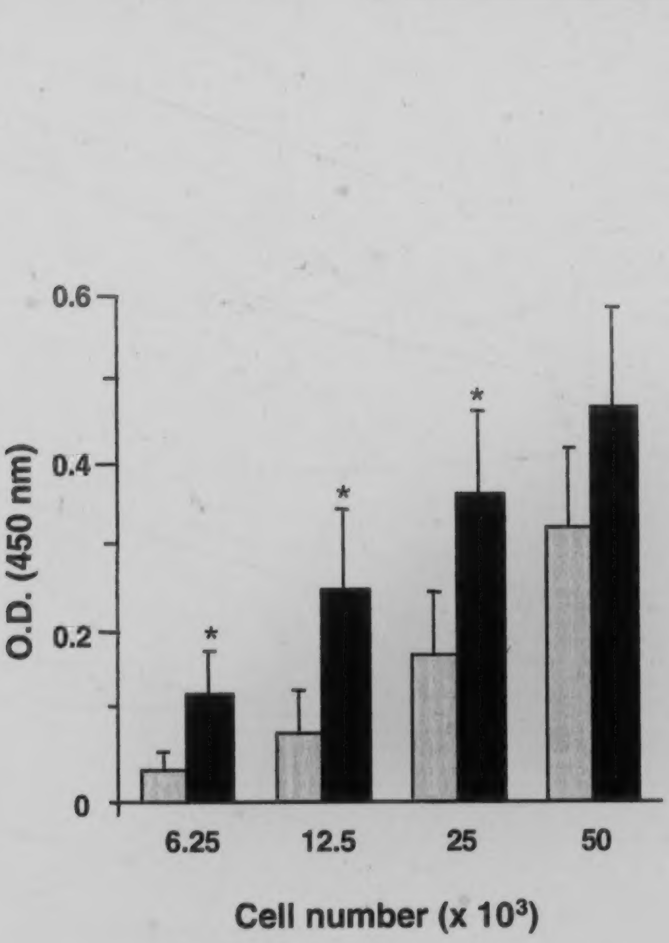

Figure 3.1: Induction of goldfish kidney leukocyte growth by GMCL-derived supernatants. Varying numbers of kidney leukocytes were seeded in wells of a 96-well culture plate and cultured in the presence (black) or absence (gray) of 25% (v/v) GMCL-derived CCM. Cell proliferation was indirectly determined after 8 days of cultivation using the XTT viability assay. Each bar represents the mean ± SEM of quadruplicate cultures established from 8 fish. Significant differences between cells grown in the presence or absence of GMCL-derived CCM ($P < 0.05$) are marked by an asterisk; differences are based on same group comparisons.

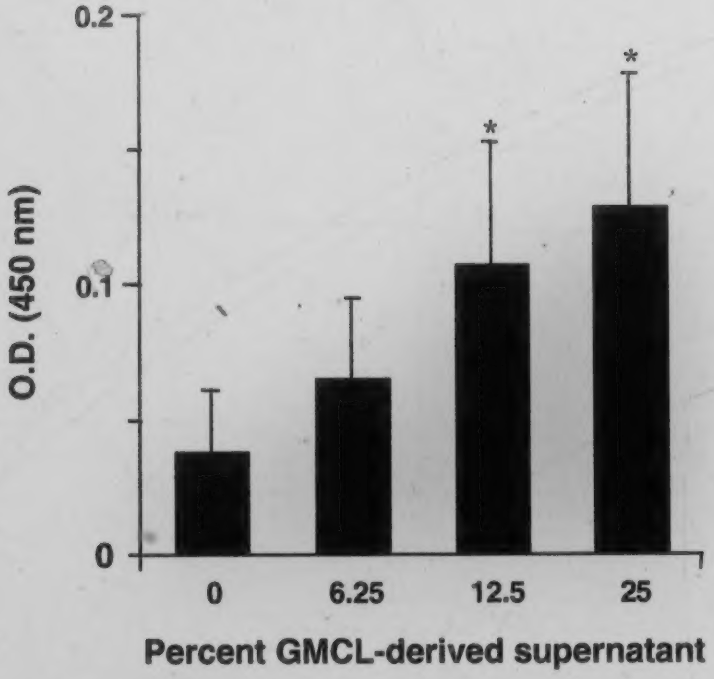

Figure 3.2: Dose-dependency of goldfish kidney leukocyte growth on GMCL-derived supernatants. 6.25×10^3 kidney leukocytes were seeded into 96-well culture plates and stimulated to proliferate using different concentrations of GMCL-derived CCM (%v/v). Cell proliferation was indirectly determined after 8 days of cultivation using the XTT viability assay. Each bar represents the mean ± SEM of quadruplicate cultures established from 8 fish. Significant differences between cells grown in the presence or absence of GMCL-derived CCM ($P < 0.05$) are marked by an asterisk.

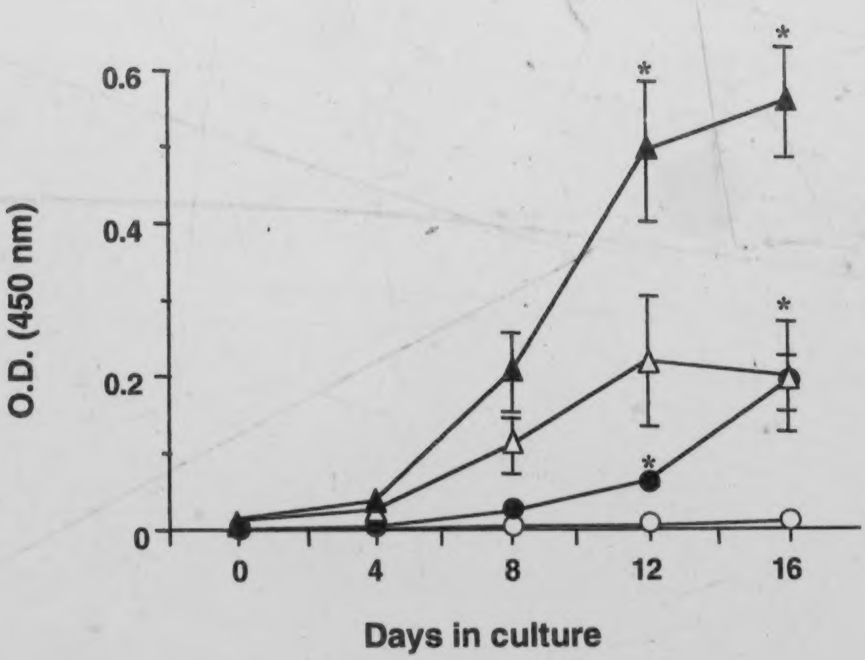

Figure 3.3: Kinetics of kidney leukocyte proliferation in response to GMCL-derived supernatants. Wells of a 96-well culture plate were seeded with 6.25x10^3 kidney leukocytes (circles) or 2.5x10^4 kidney leukocytes (triangles), and induced to proliferate in the absence (open symbols) or presence of 25% (v/v) GMCL-conditioned supernatants (filled symbols). Cell proliferation was indirectly determined after 8 days of cultivation using the XTT viability assay. Each data point represents the mean O.D. ± SEM of quadruplicate samples from 4 fish (for the 6.25x10^3 cell/well group) or 8 fish (for the 2.5x10^4 cells/well group). Significant differences between cells grown in the presence or absence of GMCL-derived CCM ($P < 0.05$) are marked by an asterisk; differences are based on same group comparisons at that specific time point.

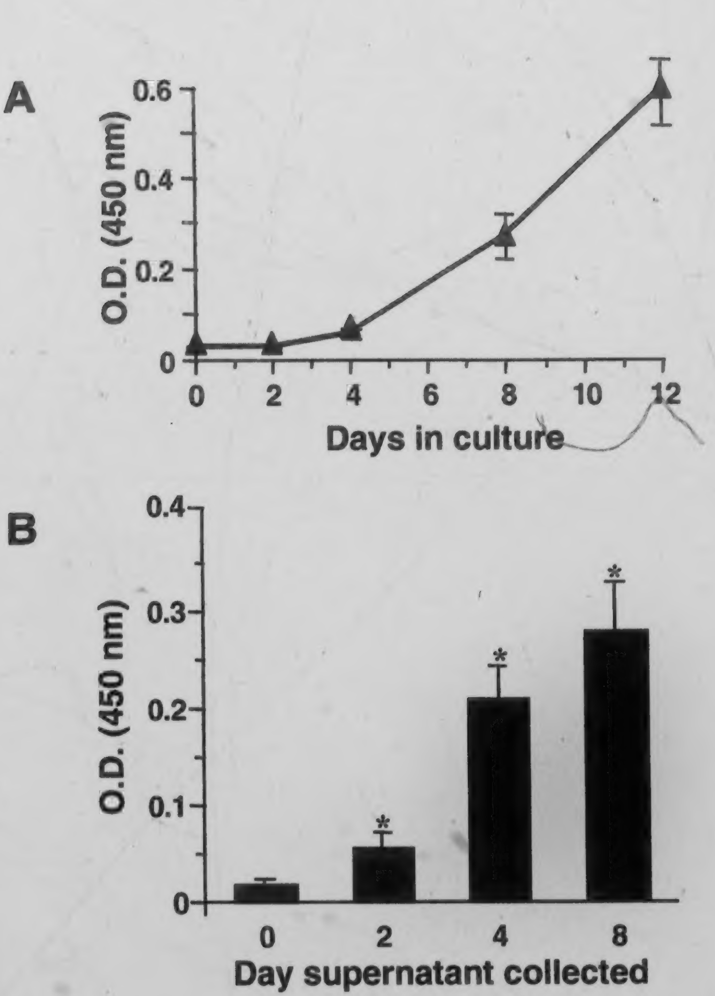

Figure 3.4: Kinetics of kidney leukocyte growth at high cell density. Proliferation of 1×10^5 kidney leukocytes seeded into wells of a 96-well culture plate (A) was indirectly assessed using the XTT viability assay. Supernatants from the cultures in graph (A) were removed at the specified time intervals indicated in (B), and tested for their ability to induce proliferation of subsequent cultures seeded with only 1.25×10^4 kidney leukocytes (B). Cell growth in (B) was determined using the XTT viability assay after 12 days of cultivation. Each data point represents the mean O.D. ± SEM of quadruplicate cultures established from 8 fish. Significant differences between cells grown in the presence of KL-derived CCM and Day 0 controls ($P < 0.05$) are marked by an asterisk.

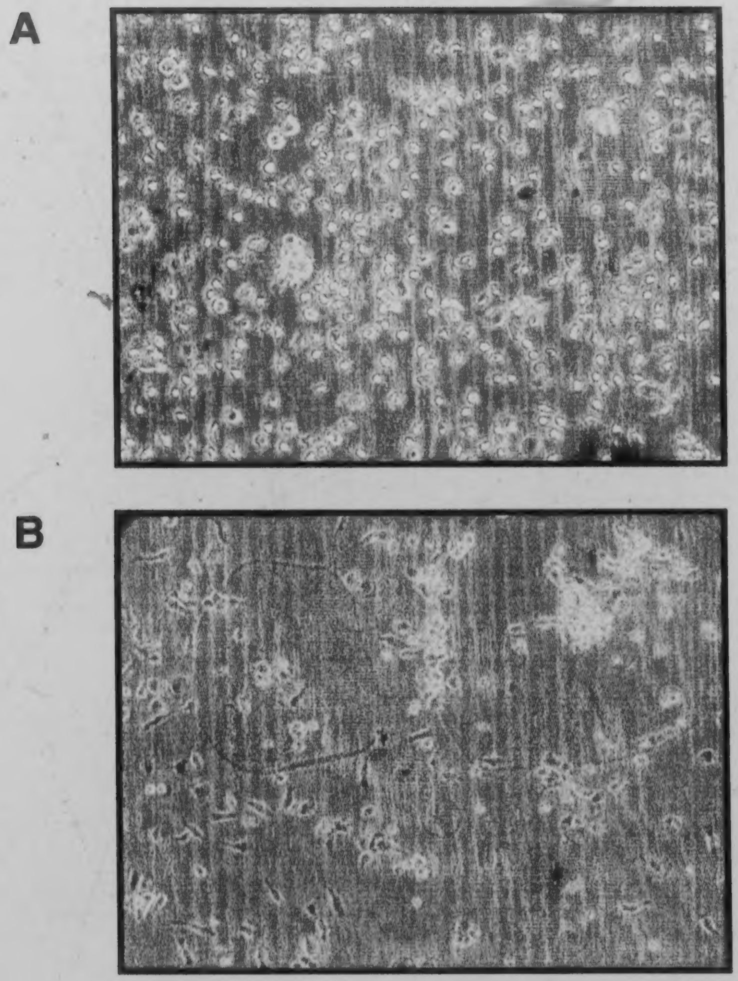

Figure 3.5: Phase contrast photomicrographs of 8-day old primary kidney macrophage cultures grown in the presence of KL-derived supernatants. Photomicrograph (A) demonstrates the general appearance of PKM cultures containing both adherent and non-adherent cells. In photomicrograph (B), the non-adherent population was removed to demonstrate the characteristic morphology of the adherent cell population. Note the appearance of small irregular cells, fried egg cells, and multinucleated giant cells in the adherent population.

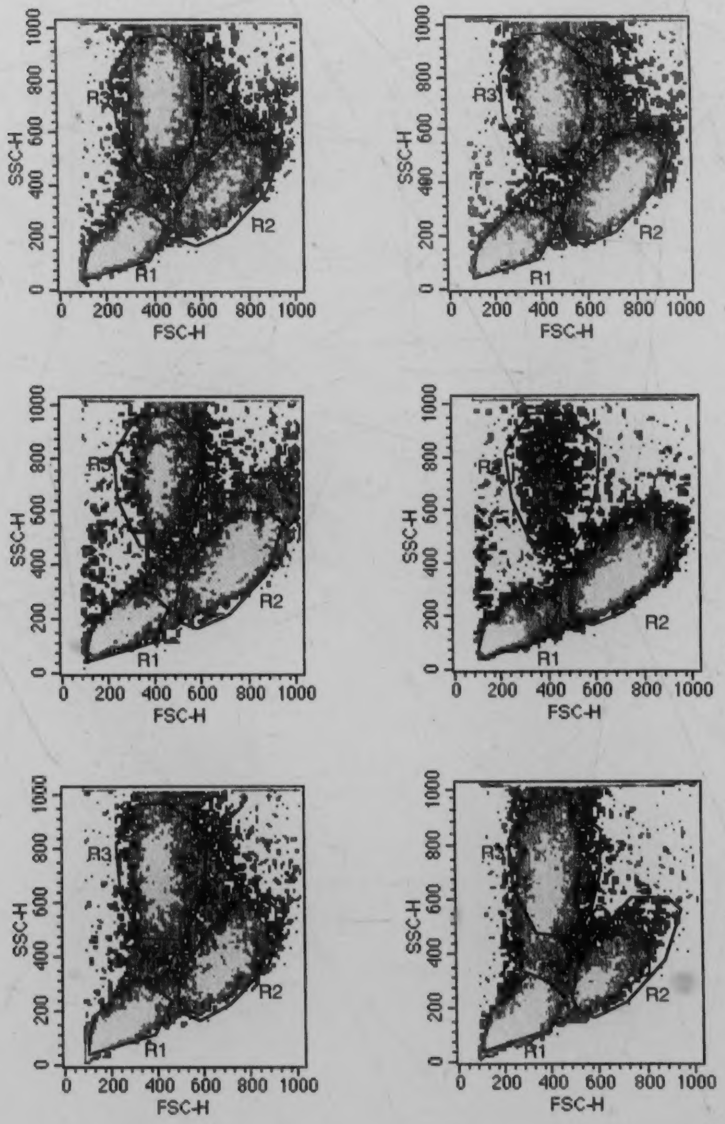

Figure 3.6: Flow cytometric density profiles of primary kidney macrophage cultures obtained from six fish. Cultures were established by seeding 2x10^7 kidney leukocytes into 75 cm^2 tissue culture flasks in the presence of 25% (v/v) kidney leukocyte-derived cell conditioned medium (KL-derived CCM). Flow cytometric analysis was performed on the non-adherent cell fraction after 8 days of cultivation; analysis of the adherent cell fraction also showed the three distinct subpopulations (data not shown). Macrophages segregating to the various regions based on size (FSC-H) and internal complexity (SSC-H) parameters were designated as R1, R2, and R3-type macrophages. Light colored areas correspond to greater cell densities.

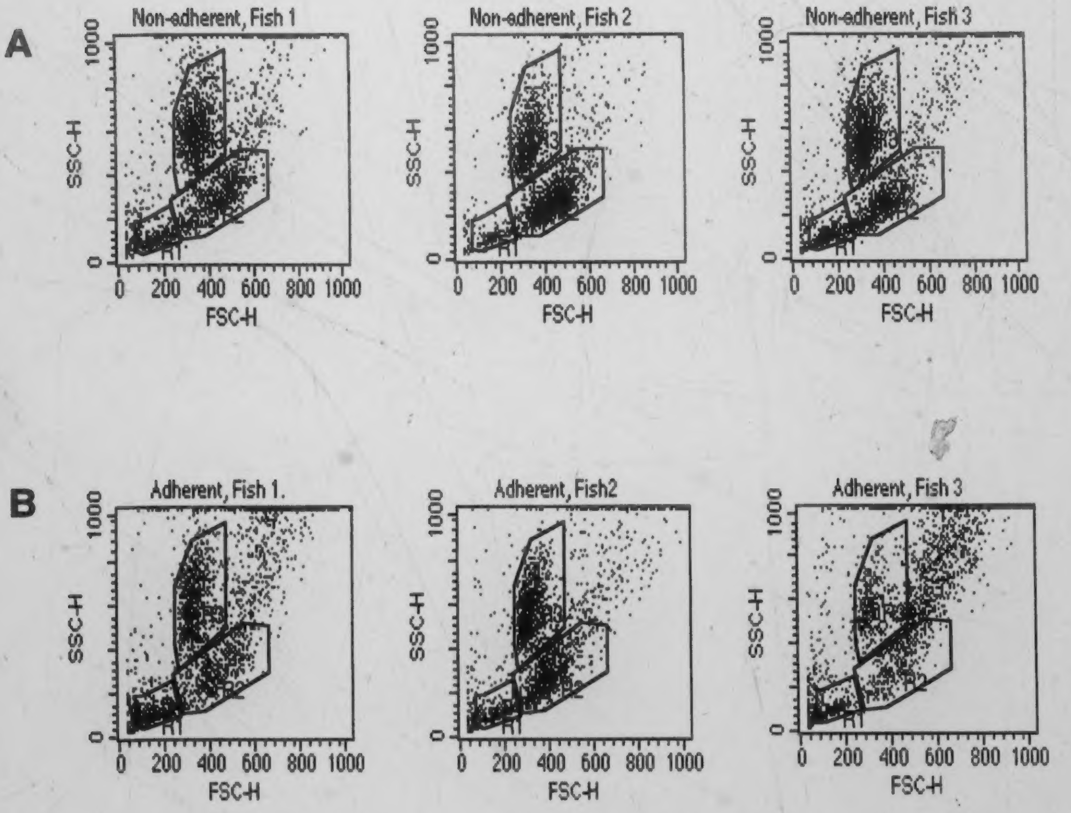

Figure 3.7: Flow cytometric analysis of adherent and non-adherent cells in 8-day old primary kidney macrophage cultures. Non-adherent cells (panel A) were removed by shaking PKM cultures and pipetting off culture media containing the non-adherent cell fraction. Adherent cells were removed by trypsinization (panel B). The data presented above show the flow cytometric profiles of adherent and non-adherent cell fractions from 3 fish. Note that the three sub-populations of macrophages (R1, R2, and R3) are present in both the adherent and non-adherent cell fractions. There is a proportion of cells that display FSC-H vs. SSC-H characteristics that place them outside the denoted gates, particularly in the top-right corner of the flow cytometric profiles; this is a common occurrence even in the non-adherent fraction, but represents a small percentage of the examined events (average 3.84% for ten primary macrophage cultures). These events appear to result from increased cellular clumping in cultures as cultivation time increases, or formation of multinucleated cells, particularly in the adherent fraction where they have been removed by trypsinization.

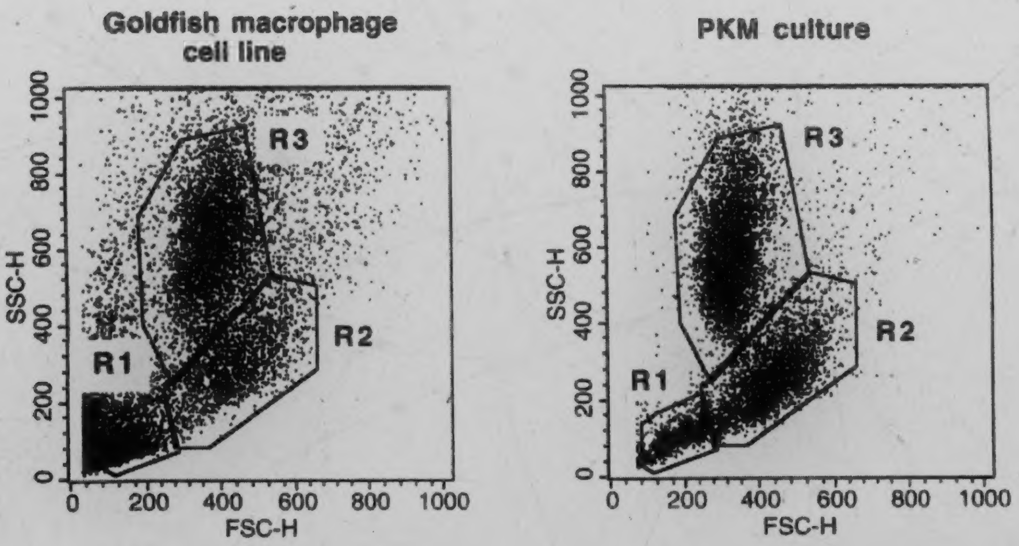

Figure 3.8: Flow cytometric profile of GMCL and 8-day old PKM cultures. Characterization of our long-term macrophage cell line, GMCL, demonstrated the presence of the same three distinct subpopulations observed in primary kidney macrophage cultures.

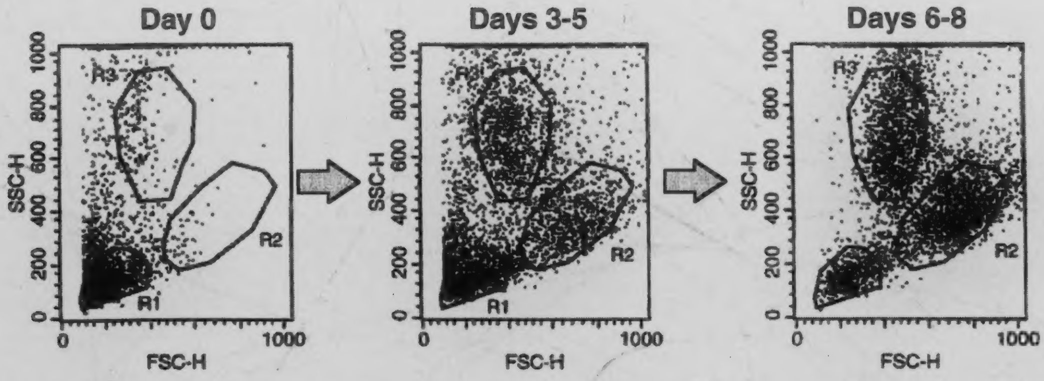

Figure 3.9: Progression of primary kidney macrophage development as determined by flow cytometry. Note the progression from a culture mainly composed of R1-type macrophages at Day 0 to one composed of three distinct subpopulations at Days 6-8. PKM cultures from twenty fish were observed; results for a representative replicate are shown.

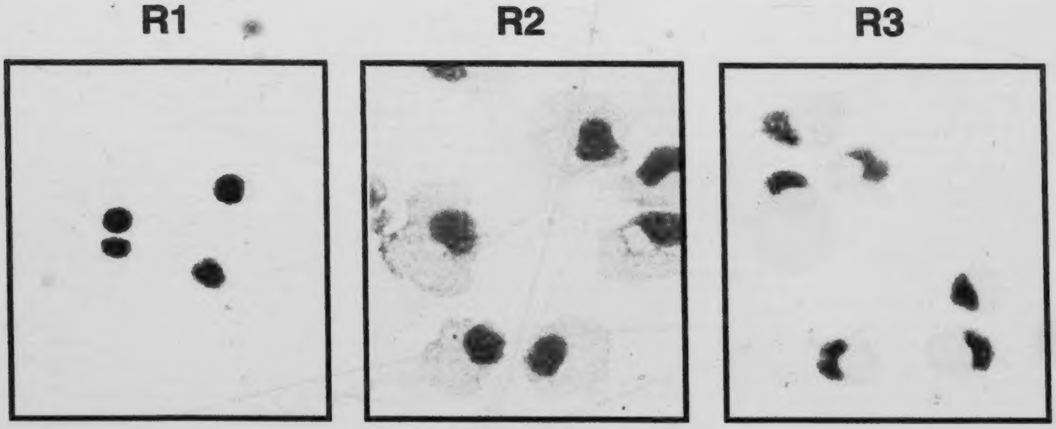

Figure 3.10: Morphological analysis of the three distinct subpopulations identified in PKM cultures. Typical Day 8 PKM cultures were analyzed by flow cytometry and each macrophage subpopulation sorted based on size and internal complexity parameters. Microscopic analysis of modified Wright's stained-cytospin preparations determined the typical morphology of cells in each subset. Representative photomicrographs are shown.

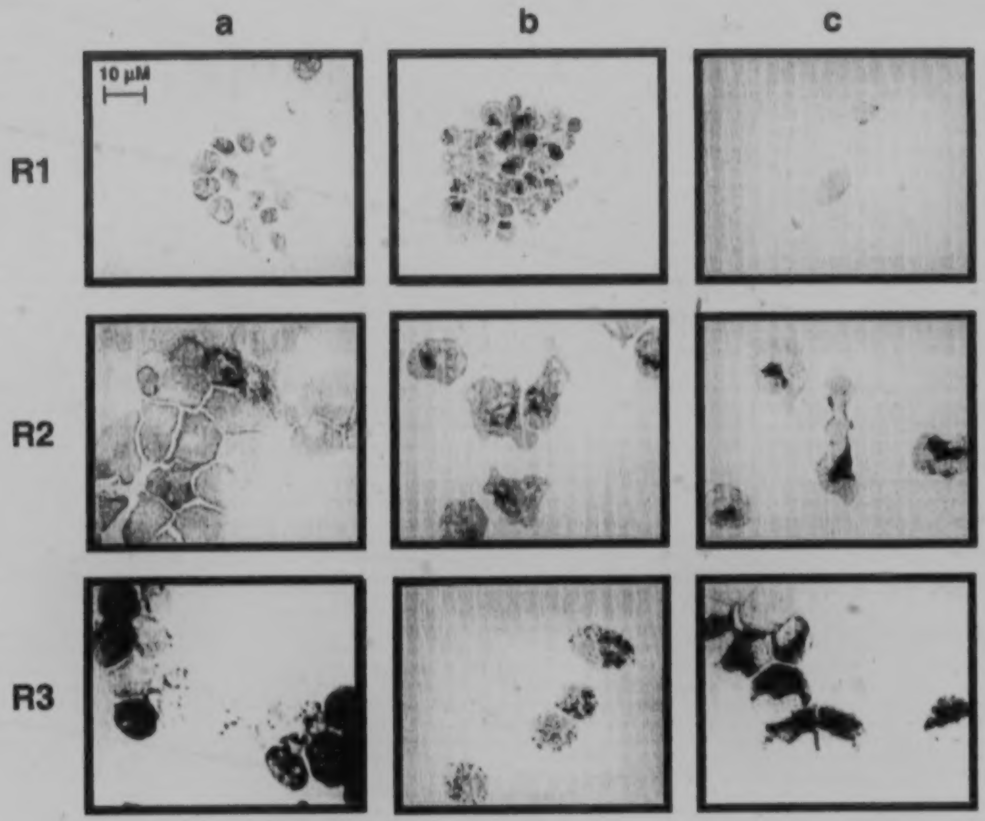

Figure 3.11: Cytochemical analysis of the three distinct subpopulations identified in PKM cultures. Typical Day 8 PKM cultures were analyzed by flow cytometry and each macrophage subpopulation sorted based on size and internal complexity parameters. Microscopic analysis of a) non-specific esterase, b) acid phosphatase, and c) myeloperoxidase stained-cytospin preparations determined the typical cytochemistry of cells in each subset. Representative photomicrographs are shown.

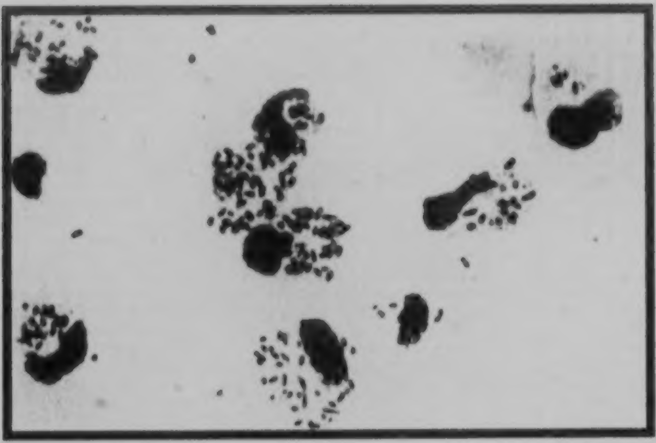

Figure 3.12: Phagocytosis of the bacterium *Aeromonas salmonicida* by 8-day old primary kidney macrophages.

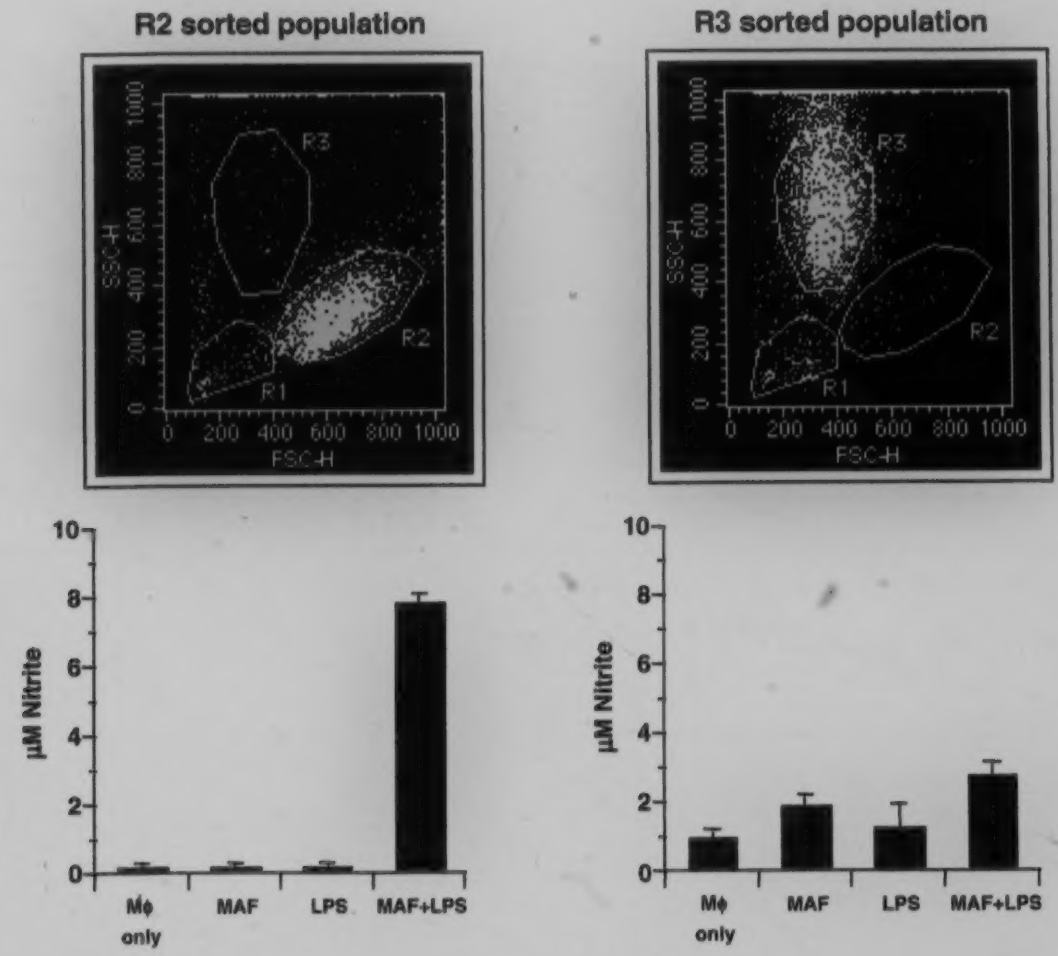

Figure 3.13: Production of nitric oxide by R2 and R3-type primary kidney macrophages. R2 and R3-type macrophages were sorted using flow cytometry, seeded into wells of half-area 96-well culture plates (2.5x10^4 cells/well), and stimulated to produce nitric oxide in response to stimulation with MAF (1:4 dilution) and LPS (1 µg/ml). Nitrite produced by activated macrophages was determined after 72 hrs. Each data point represents the mean ± SEM of triplicate cultures. Two experiments were performed; representative results are shown.

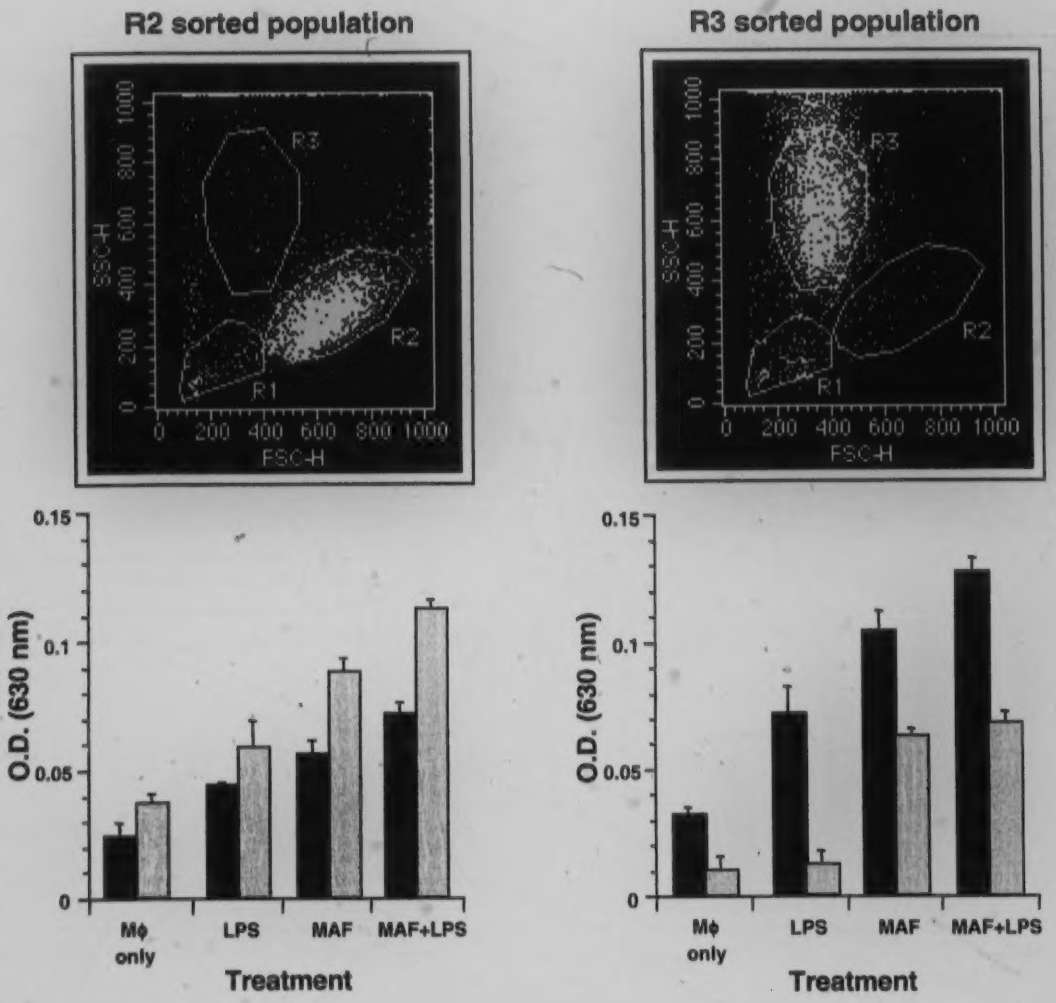

Figure 3.14: Priming of the respiratory burst response of R2 and R3-type macrophages with MAF and/or LPS. Macrophage subpopulations were sorted from primary kidney macrophage cultures using flow cytometry and seeded into wells of half-area 96-well culture plates (2.5×10^4 cells/well). Macrophages were stimulated with MAF (1:3 dilution) and/or LPS (1 µg/ml) for 6 hrs (black) or 48 hrs (gray). PMA-triggered respiratory burst responses were determined using the NBT assay. Each bar represents the mean ± SEM of triplicate cultures. Two experiments were performed; representative results are shown.

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CHAPTER IV: FLOW CYTOMETRIC ANALYSIS OF PROLIFERATION AND DIFFERENTIATION IN GOLDSFISH KIDNEY-DERIVED MACROPHAGES[3]

1. INTRODUCTION

Macrophage renewal is generally attributed to hematopoiesis, the generation of mature blood elements as a result of early progenitor development (1). In mammals, this process, according to the mononuclear phagocyte system theory, encompasses a complex series of events involving the differentiation of multipotential stem cells to committed progenitor cells in the bone marrow (2-4). Bone marrow progenitor cells proliferate and differentiate to blood monocytes, which migrate and enter the tissues in response to local stimuli. Once in the tissues they undergo site-specific maturation to become fully-differentiated tissue macrophages. Recent advances in the field of macrophage hematopoiesis, however, have expanded this theory. Of particular interest are the observations that: 1) macrophage heterogeneity can be generated through alternate paths of differentiation, and 2) self-renewal of resident macrophages may be responsible for the re-population of diminished tissue macrophage subsets.

Macrophages represent a heterogeneous population of cells that can be distinguished based on their location in the vertebrate body (5, 6), morphology (7-10), cytochemistry (8-11), and function (8, 9, 11-16). This heterogeneity has been attributed to a finely interwoven network of positive and negative regulators of macrophage development, including soluble mediators (1, 17-22) and a variety of microenvironmental conditions (23-28). Early studies recognized the existence of two different macrophage populations: M-CSF and GM-CSF-derived macrophages (29-32). More recently, several studies have described the development of several distinct macrophage populations as a result of lineage divergence at different stages throughout the macrophage differentiation pathways (7, 11, 13, 16, 33). Such divergence into alternate paths of differentiation allows for the generation of exquisitely specialized macrophage subsets necessary to maintain host immunocompetence and homeostasis.

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[3] A version of this chapter has been published:
Barreda, Neumann, and Belosevic (2000) *Dev. Comp. Immunol.* 24(4): 395-406.
D.R. Barreda was involved in the data collection, analysis, and writing of the manuscript.

Consistent with the mononuclear phagocyte theory, renewal of resident macrophage populations has been generally attributed to the regular influx and subsequent maturation of blood monocytes rather than local proliferation (2, 4, 34). The proliferative capabilities of mature resident macrophages, however, remain unclear for even in these early studies proliferative activities of up to 5% were observed (2, 4). More recently, a number of studies point to a greater potential contribution of such renewal. For example, it has been found that elimination of circulating monocytes, by Strontium-89 treatment, does not affect the total numbers or proliferative potential of resident macrophage populations in the peritoneum, lungs, or liver (34-36). Moreover, microglia-specific progenitor cells have been identified within the brain, a site not readily accessible for re-population via influx of monocytes, due to the blood-brain barrier (37). These data suggest that resident macrophage populations in various tissues are capable of self-renewal in the absence of the "classical" hematopoietic-derived pathways of macrophage renewal where monocytes play a key role.

Recent observations warrant the consideration of similar issues in macrophage heterogeneity and self-renewal in fish macrophage systems. A goldfish (*Carassius auratus*) macrophage cell line (GMCL) and primary kidney macrophage (PKM) cultures have been established and characterized in our laboratory [(38), Chapter III]. Flow cytometric analysis of both these culture systems indicated the presence of three distinct macrophage sub-populations (Chapter III). Functional, morphological, cytochemical, and flow cytometric characterization of PKM subpopulations suggested that these three subsets represent cells of the macrophage lineage, but at distinct differentiation junctures of their development.

In this chapter, I describe the characterization of proliferation and differentiation events leading to the generation of mature macrophages from early fish macrophage progenitors using a flow cytometric approach. I first describe the development of FACS-sorted macrophage subsets in response to endogenous macrophage growth factors (MGF). Subsequently, a non-radioactive, fluorescent lipid membrane-bound dye, PKH26

(The Cell Census Plus™ System, Sigma) (39) was used to examine the development of primary kidney macrophages in the context of three continuously interacting macrophage subsets, each at a distinct stage of fish macrophage differentiation. Flow cytometric analysis of PKH26-labeled PKM cultures allowed for quantitative assessment of macrophage proliferation *in vitro*. In addition, PKH26 was used to track the putative differentiation pathways of cells within PKM cultures.

2. RESULTS AND DISCUSSION

Continuous renewal of macrophages and differentiation into functionally mature effector cells is necessary for host defense and the maintenance of homeostasis (17, 40, 41). In Chapter III we reported that goldfish macrophages secrete an autocrine growth factor that induced the selective proliferation of macrophages from goldfish kidney leukocyte cultures. Similarly, the production of an endogenous macrophage growth factor has been described in rainbow trout (42).

Macrophages that develop in PKM cultures represent a heterogeneous population of cells, as determined by flow cytometry (designated by us as R1, R2, R3 subpopulations; Fig. 4.1). Further analysis of the morphological, cytochemical, functional, and developmental characteristics of the three distinct PKM subpopulations suggested that these might represent cells arrested at distinct differentiation junctures in the macrophage lineage (putative early progenitors, monocytes, and "mature" macrophages) (Chapter III). Moreover, we have demonstrated the presence of such subpopulations in a goldfish macrophage cell line established and propagated in the laboratory over the past 7 years. R1 cells are the first to appear in PKM cultures, and comprise small cells (6-10μm) with high nucleus to cytoplasm ratio (Fig. 4.1). We have defined the R1 subset as "early progenitors" based on expression of c-kit (Fig. 7.8 Chapter 7), which encodes for the stem cells factor receptor, commonly used as a marker of stem cells and other early hematopoietic progenitors. R3 cells, putative monocytes, are large round cells (12-15μm) with eccentrically placed kidney-shaped nuclei and low nucleus to cytoplasm ratio (Fig. 4.1). This subpopulation contains acid phosphatase, diffuse cytoplasmic myeloperoxidase, and non-specific esterase. R2 cells are morphologically similar to mature tissue macrophages of mammals and contain acid phosphatase, localized nuclear myeloperoxidase, and non-specific esterase. Both R3-monocytes and R2-mature macrophages were shown to exhibit distinct nitric oxide and respiratory burst responses (Chapter III). The present study was undertaken to examine the proliferation and differentiation events leading to the generation of mature macrophage-like cells from early progenitor-like cells isolated from goldfish kidney hematopoietic tissues.

The three macrophage subpopulations were sorted using the flow cytometer, and their proliferative potential examined. We chose to use FACS-sorted macrophage subpopulations as our previous attempts to clone individual cells within each of these subpopulations were not successful.

R1 early progenitor-like cells appeared to differentiate into R2 mature macrophage-like cells over 4-8 days of cultivation (Fig. 4.2). Moreover, proliferation of R1-early progenitors occurred over the 4-8 days observation period in the presence or absence PKM-derived cell conditioned medium (CCM); however, proliferation of unstimulated R1-early progenitors was slower compared to those stimulated with PKM-derived CCM (Table 4.1).

R2- macrophages appeared to be a more mature macrophage subpopulation, in that purified R2 cells remained in their sort region after 4 days of cultivation in the presence or absence of exogenously added PKM-derived CCM (Fig. 4.3). Like R1-early progenitors, the R2-mature macrophage subpopulation proliferated significantly in the presence of PKM-derived CCM (Table 4.1); in contrast, R2-mature macrophage numbers did not increase significantly in the absence of PKM-derived CCM. At present, it is unclear whether this was a function of their inability to produce sufficient amounts of MGF to induce self-renewal, or the requirement of additional factors to promote R2-mature macrophage survival and/or to co-stimulate proliferation events.

In contrast to R1-early progenitors and R2-mature macrophages, purified R3 monocyte-like cells were unable to proliferate in the presence or absence of PKM-derived cell conditioned medium (Table 4.1). However, addition of PKM-derived CCM appeared to maintain the viability of treated cells, as the number of R3-monocytes cultured in the absence of PKM-derived CCM decreased significantly over the observation period and was further correlated with increased debris formation (Table 4.1 and Fig 4.4). In the presence of PKM-derived CCM, R3-monocytes shifted out of their FACS-defined gate and partially migrated towards the R2-mature macrophage gate (Fig. 4.4). This may represent a differentiation event. Alternatively, this may be analogous to the R3-monocyte shift observed between Days 8 and 13 (Fig. 4.7), which we have correlated with PKM entrance into a senescent state (discussed in detail in Chapter V). The accurate determination of the physiological events represented by this flow cytometric shift must await the generation of differentiation stage-specific antibodies to be used in the characterization of PKM subsets and in the establishment of improved protocols for their isolation.

After numerous attempts we were unable to effectively clone individual cells from each PKM subpopulation. This was particularly intriguing for cells of the R1-early progenitor and R2-mature macrophage subsets, as they are capable of proliferating in the absence of exogenous growth factors. Additional regulatory cues may be necessary to activate proliferation events, and could include soluble mediators or paracrine modulators that require the presence of significant cell numbers ($n > 1$) for the induction of PKM growth.

Analysis of the proliferation and differentiation of sorted PKM subpopulations (described above) pointed to the contrasting intrinsic abilities of each subset to support developmental events. In order to examine proliferation and differentiation events in the context of whole PKM cultures, where there was continuous interaction between developing macrophages at subsequent stages of differentiation, we decided to use a PKH26 labeling approach. PKH26-labeled cells fluoresced significantly more intensely than unlabeled cells (Fig. 4.5), and labeling with PKH26 did not affect PKM viability, since both labeled and unlabeled cells displayed similar flow cytometric profiles throughout culture development (Fig. 4.6). PKM actively proliferated in culture yielding 7-8 generations through the observation period. This resulted in a gradual decrease in the mean fluorescence intensity of the cells (Fig. 4.7), which was indicative of proliferation in the cultures. The relative percentages of each generation for a representative culture at the various time points were tabulated in Table 4.2. Interestingly, as PKM proliferated in culture the fluorescence histograms broadened, which was indicative of a heterogeneous cell population not dividing synchronously (Fig. 4.7)

As early as 3 days after cultivation, a shift of the parental population from R1 to R3 and R2 occurred (compare parent generation [G.P.] Day 0 to G.P. Day 3, Fig. 4.8). The shift from R1 to R3 (early progenitor→monocyte) was consistent with the differentiation of early progenitors along the classical pathway of hematopoiesis in which monocytes play a key role. This was also consistent with our earlier observations demonstrating that FACS-purified R3 monocyte-like cells appeared to differentiate into R2 macrophage-like cells when cultured in the presence of exogenous growth factors (Fig 4.2). The R1 to R2 shift (early progenitor→macrophage) suggested the presence of an alternate pathway of differentiation towards a mature macrophage stage in the absence of an intermediate monocyte-like stage. One should not disregard the possibility that such shifts early in the macrophage development may represent a change in morphology of newly seeded cells that occurred as they were transferred from the kidney to *in vitro* conditions. The change in growth conditions may lead to a change in morphology and, hence, flow cytometric characteristics within the first 24 hours of cultivation. However, several cultures capable of R3-monocyte development did not exhibit such a subset until later in the macrophage development (Day 6-8) (data not shown). Moreover, we have previously shown that sorted R1 cells, obtained from *in vitro* cultures (> 6 days old) shifted towards and localized within the R2 gate (Fig. 4.2). Therefore, our data suggested that these events represent differentiation rather than morphological changes as a result of growth condition differences. In addition, the shift to R2 and R3 within the parent generation suggested that proliferation may not be a necessary event prior to the differentiation of early progenitors to more mature stages along the mononuclear phagocyte pathway in fish. A summary of the postulated alternative pathways of differentiation leading to the generation of mature macrophages from early progenitors in PKM cultures was shown in Fig. 4.1.

The R1, early progenitor-like subset, appeared to be a self-proliferating subpopulation; note progressive generations within the R1 subset (Fig. 4.8). By Day 4, three generations (i.e. R1 was present in G.P., G.2, G.3; Fig. 4.8) are easily distinguished in our analyses, supporting our earlier observation that sorted R1 cells increased in number after cultivation (Table 4.1). Early progenitors did not proliferate synchronously (see decreasing non proliferative fraction in Table 4.2). Instead, there appeared to be a constant but limited renewal of these cells from the parent population into subsequent generations, suggesting the presence of a temporal control mechanism that regulated the proportion of early progenitors entering the path toward maturation. Such regulation was maintained throughout fish macrophage development leading to the broadening of the histograms in older macrophage cultures (Day 0-13, Fig. 4.8). In contrast, a synchronously dividing culture would be expected to yield a peak of homologous width that shifted to the left as the macrophages proliferated and matured. This temporal regulation may be a result of various microenvironmental conditions existing within the kidney, prior to the isolation of cells, as well as various systemic regulators derived from the circulation. Alternatively, this may be related to more intrinsic progenitor characteristics, a situation analogous to various mammalian systems, where early progenitor subsets with varying proliferation kinetics have been identified (43). Thus, the regulation of the number of cells entering the maturation pathway, would allow for constant control of hemopoietic resources maintaining homeostasis.

Our results suggest that R2 mature macrophage-like cells are capable of self-proliferation. On Day 5 after cultivation (Fig. 4.8), the fourth generation (G.4) of macrophages was almost exclusively comprised of mature cells. On Day 6 after cultivation, we observed the fifth generation of cells, and the appearance of this generation can only be explained by postulating cellular division of macrophages of the fourth generation. Logically, Day 6 (G.5) cells must have developed from the proliferation of macrophages on Day 5 of cultivation (G.4). This self-renewal was clearly observed until Day 8 of cultivation (Fig. 4.8). Microscopic examination of purified R2 populations provided further support for these findings: FACS-sorted R2 cells stained using modified Wright's stain (Fisher) were found to contain several cells in the late stages of cytokinesis (data not shown).

PKM cultures appeared to be exhaustive. As the cultures approach Day 11-13 (see histograms of Fig. 4.8) the proportion of cells in the later generations progressively increased while the proportion of cells in parent and other early generations decreased. This shift towards G.8, however, was not accompanied by the appearance of subsequent generations (e.g. G.9) suggesting that the macrophages in older cultures were unable to proliferate past this stage. Microscopic observation showed various distinguishable changes in macrophage morphology consistent with descriptions of senescent cultures (44, 45). Cellular clumping, relative amount of adherent cells, and increased cellular vacuolization were some of the features observed early, and this was followed by cell death and increased debris formation at late stages. Hence, it appeared that the shift of flow cytometric profiles (Days 11-13, Fig. 4.8) was the result of the aforementioned morphological changes, characterizing the PKM cultures entering a period of senescence.

The entrance of PKM cultures into senescence as the culture reached G8, however, does not appear to be a function of the limited proliferation potential of PKM cells. Several PKM cultures expressed proliferative capabilities beyond the eight-generation, proliferating rapidly, even after several passages (please see discussion in Chapter III). Furthermore, the spontaneous generation of a number of long-term macrophage cultures from various fish species may be a reflection of the enormous proliferative potential of these cells (38, 42, 46-49). The cessation of PKM proliferation, shown in this chapter, may be due to the production of proliferation inhibitory factors rather than the limited proliferation potential of PKM cells. Various microenvironmental conditions (including cell density, nutrient deprivation, cell size) may lead to the activation of negative feedback loops and the production of negative regulators of proliferation geared to the maintenance of homeostasis/culture integrity. In contrast, the elimination or delay of such conditions may lead to increased proliferation and culture growth characteristic of highly proliferative cultures allowing for the establishment of long-term macrophage cell lines. At present, the identity of such inhibitory factors and the mechanisms leading to their release remain unknown.

The detailed characterization of the various developmental stages in PKM cultures, and the various proliferation and differentiation events involved in PKM development will allow for a more precise examination of the hematopoietic pathways involved in fish macrophage development, and will contribute significantly to our understanding of the biology of fish macrophages and the mechanisms involved in the host defenses of fish to infectious agents.

Table 4.1: Proliferation of FACS-sorted R1, R2 and R3-type macrophages in the presence or absence of primary kidney macrophage-derived cell conditioned supernatants.[a]

Cell Type	Culture Conditions	Days in Culture	Number of cells per well ± SEM (x10^4)
R1	PKM-derived CCM present	0	1.24 ± 0.10
		4	1.21 ± 0.06[c]
		8	4.42 ± 0.24[b,c]
	PKM-derived CCM absent	0	1.22 ± 0.07
		4	0.85 ± 0.06[b]
		8	3.14 ± 0.05[b,c]
R2	PKM-derived CCM present	0	2.56 ± 0.09
		2	2.58 ± 0.17
		4	3.93 ± 0.25[b,c]
	PKM-derived CCM absent	0	2.56 ± 0.09
		2	2.53 ± 0.13
		4	2.73 ± 0.060
R3	PKM-derived CCM present	0	2.50
		2	2.52 ± 0.06
		4	2.34 ± 0.13[b]
	PKM-derived CCM absent	0	2.50
		2	2.23 ± 0.04[b]
		4	1.83 ± 0.03[b,c]

[a] R1, R2 and R3-type macrophages were sorted using flow cytometry and seeded into wells of half area 96-well culture plates, 1.25x10^4 cells (R1) and 2.5 x 10^4 cells (R2 or R3). The cells were cultured in the presence or absence of PKM-derived CCM. The number of macrophages in the culture wells was determined on the specified days using flow cytometry.

[b] The number of macrophages in culture is significantly different compared to Day 0 cultures for the same treatment group ($P < 0.05$).

[c] The number of macrophages in the PKM-derived CCM treated group is significantly different compared to macrophage cultures without PKM-derived CCM at that same time point ($P < 0.05$).

Table 4.2: Proliferation of goldfish kidney-derived macrophages labeled with PKH26 fluorescent dye.[a]

A)

Day	Number of Generations	Proliferation Index	Non-proliferative fraction
0	1	1	N/A
3	2	1.24	0.58
4	3	1.57	0.36
5	4	1.88	0.30
6	5	2.60	0.19
8	6	3.54	0.16
11	8	8.65	0.05
13	8	12.78	0.03

B)

Day of Cultivation	Percent of Macrophages in Each Generation G.P.	G.2	G.3	G.4	G.5	G.6	G.7	G.8
0	84.2	----	----	----	----	----	----	----
3	57.9	36.7	----	----	----	----	----	----
4	36.3	39.0	20.7	----	----	----	----	----
5	30.2	21.7	36.7	7.9	----	----	----	----
6	19.3	14.8	30.6	17.1	15.8	----	----	----
8	15.7	3.6	25.2	18.9	20.5	14.7	----	----
11	4.6	3.1	7.6	8.5	21.5	19.7	23.9	10.7
13	3.2	1.5	5.34	4.1	13.6	15.6	20.0	35.1

[a] ModFit deconvolution of FACS-derived histograms (Figs. 4.7 and 4.8) allowed the determination of (A) the number of generations (G.P.-G.8) that appeared as the PKM culture developed, and the fraction of non-proliferating cells at each observed time point. The proliferation index was calculated as outlined in the Materials and Methods (Chapter II). G.P.= parental population G.2 to G.8= cell generations. The percentages of cells in each generation are shown in (B).

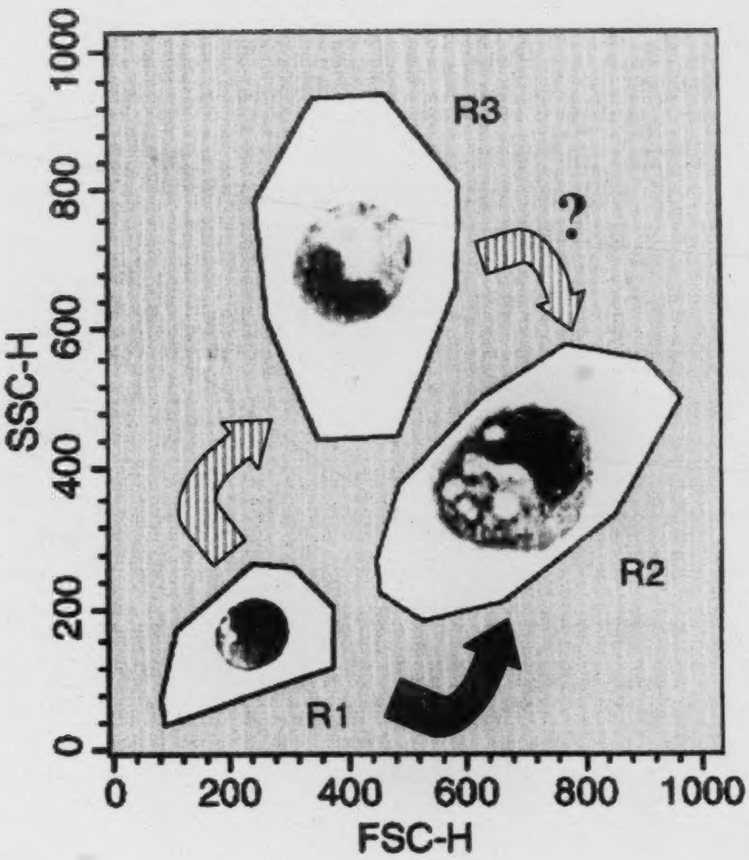

Figure 4.1: Morphology of PKM subpopulations and postulated alternate pathways of differentiation governing macrophage development (arrows). Cells from each subset were sorted using flow cytometry and stained with a Modified Wright's stain. Representative photomicrographs of cells are shown in flow cytometry gates where each of the three subpopulations segregate (designated as R1, R2 and R3-type macrophages). The PKM pathway of differentiation, that is consistent with the classical mononuclear phagocyte system theory of mammalian macrophage differentiation is indicated by hatched arrows. The alternate pathway of PKM differentiation where mature macrophages develop in the absence of a typical intermediate monocyte stage is indicated by a black arrow. The question mark indicates that it remains unclear whether the R3→R2 shifts observed represented differentiation events or were associated with entrance into a senescent state (compare to Day 13 Fig. 4.7).

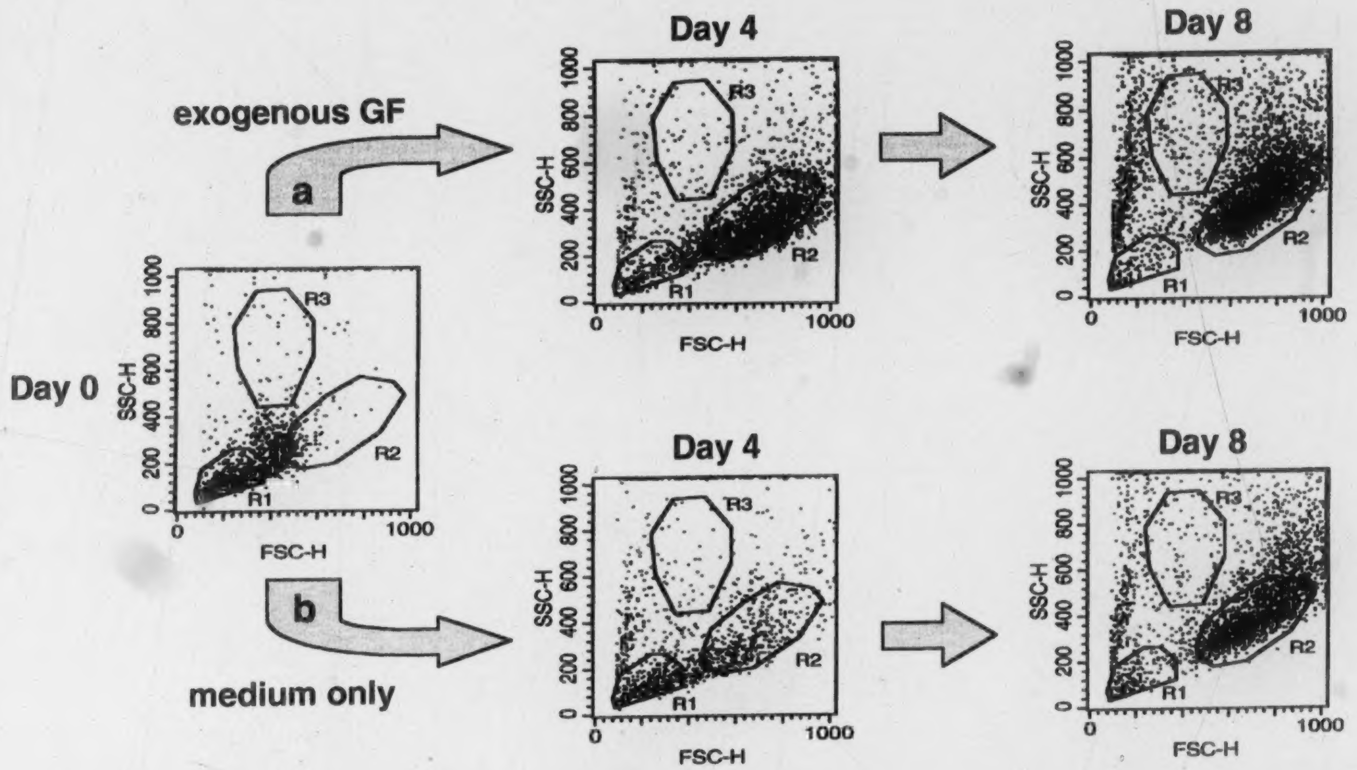

Figure 4.2: Flow cytometric analysis of sorted R1 early progenitor-like cells after cultivation in the presence or absence of primary kidney macrophage-derived cell conditioned supernatants. R1-early progenitors were sorted using flow cytometry and cultured in the presence (a) or absence (b) of PKM-derived CCM (25% v/v). Note an increase in R2-mature macrophage number after cultivation for only 4 days in cultures with PKM-derived CCM. In cultures without PKM-derived CCM the increase in the number of R2-mature macrophages was slower.

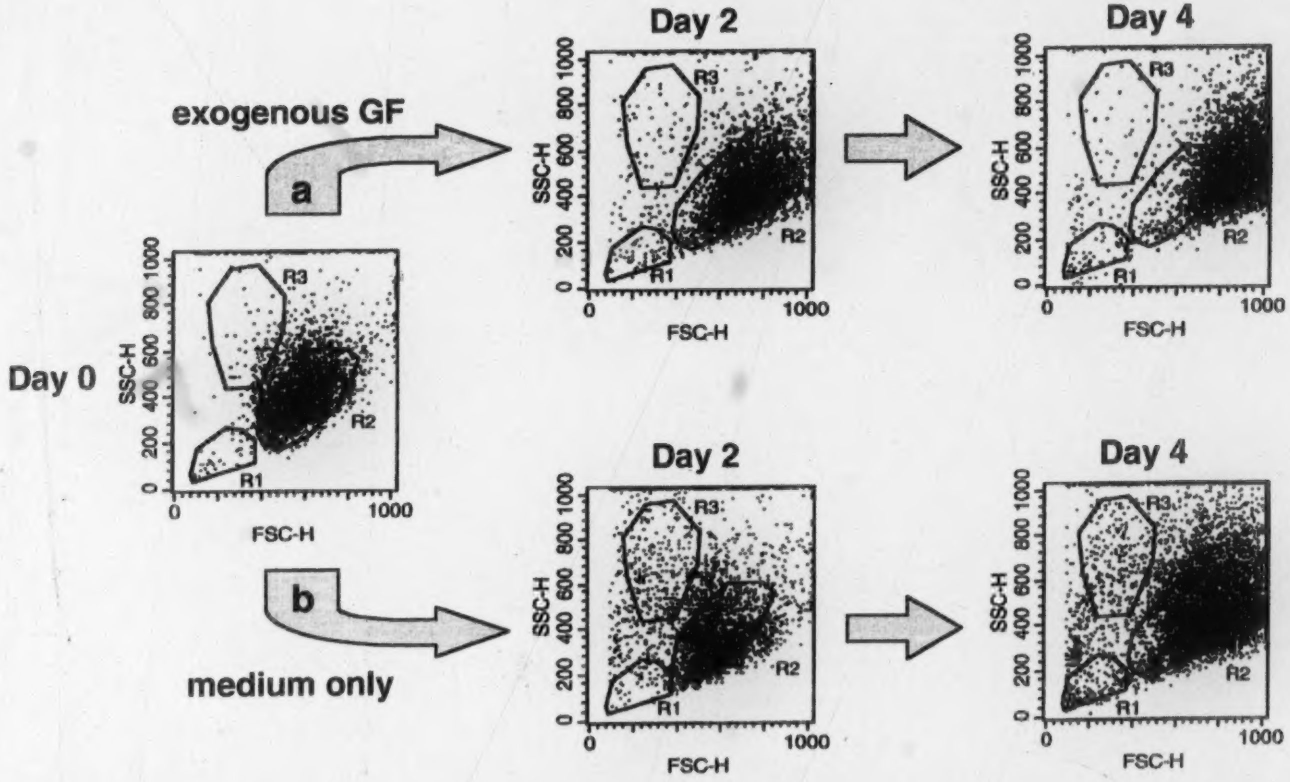

Figure 4.3: Flow cytometric analysis of sorted R2 mature macrophage-like cells after cultivation in the presence or absence of primary kidney macrophage-derived cell conditioned supernatants. R2-macrophages appear to be a more mature subpopulation in PKM cultures, since sorted cells remain in the sort region after 4 days of cultivation, regardless of whether they are cultured in the presence (a) or absence (b) of PKM-derived CCM (25% v/v).

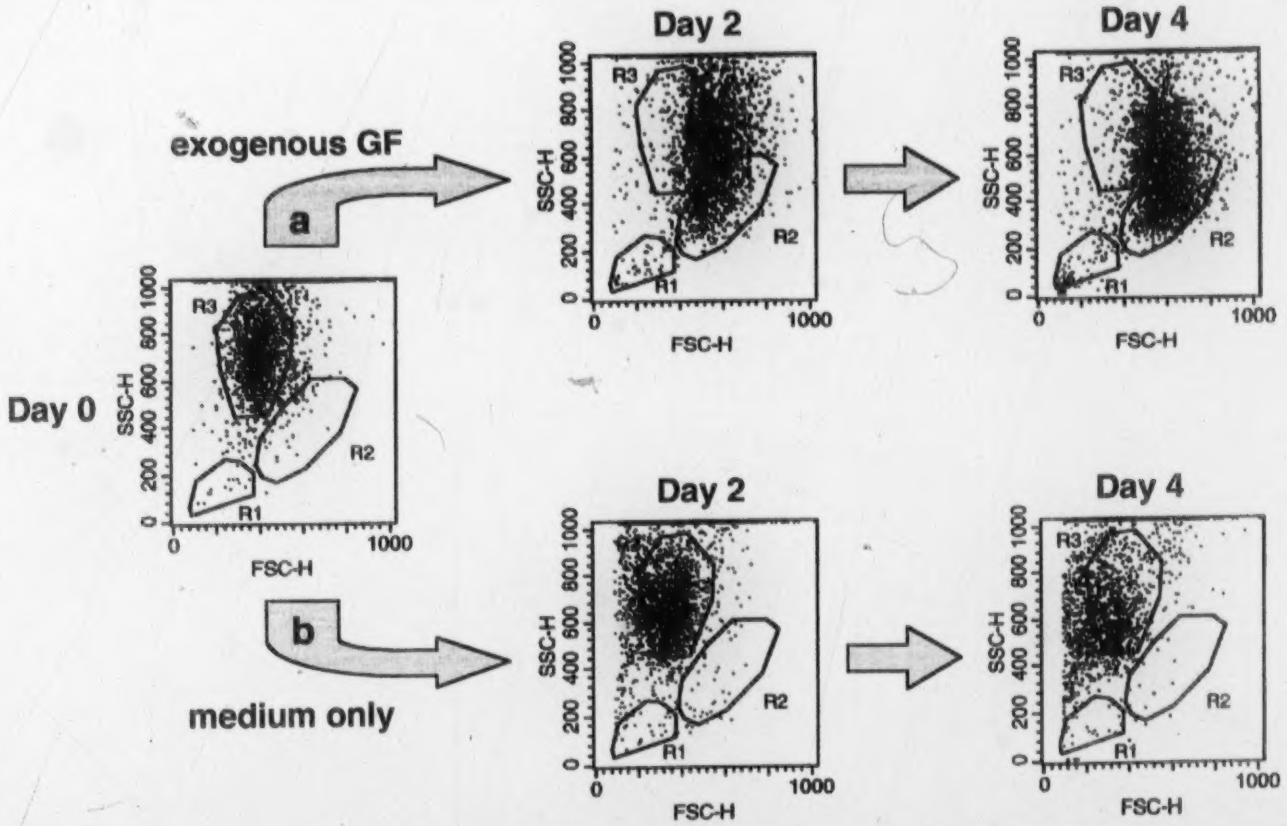

Figure 4.4: Flow cytometric analysis of sorted R3 monocyte-like cells after cultivation in the presence or absence of primary kidney macrophage-derived cell conditioned supernatants. R3-monocytes were sorted and cultured in the presence (a) or absence (b) of PKM-derived CCM (25% v/v). Differentiation of R3-monocytes into R2-mature macrophages appeared to occur only if R3-type cells were cultured in the presence of PKM-derived CCM. Moreover, R3-monocytes require PKM-derived CCM for sustained viability, as seen by a left shift in the cytometric profile of these cells by Day 4 in (b) denoting increased cellular debris.

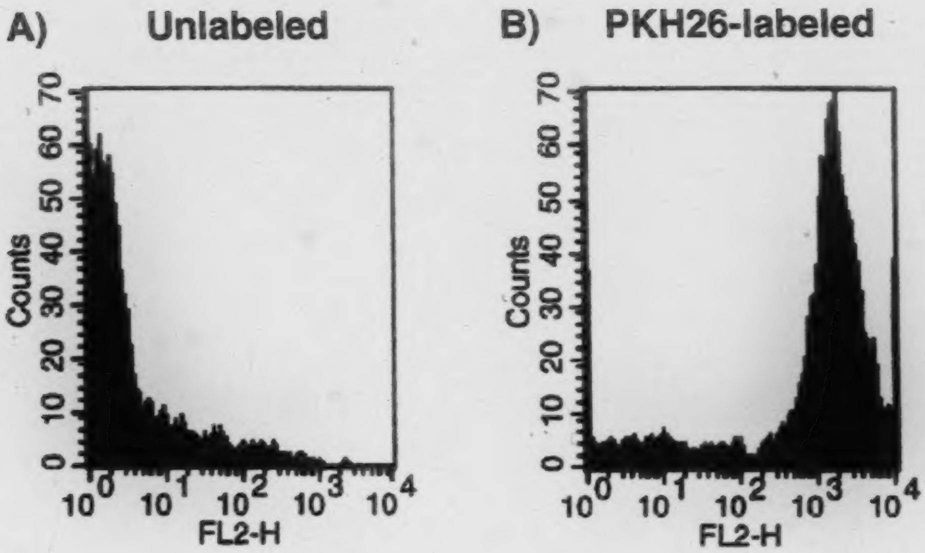

Figure 4.5: Fluorescence intensity differences between non-labeled (A) and PKH26-labeled (B) primary kidney macrophages. Fluorescence intensity was plotted on a four decade logarithmic scale. 10^4 events of triplicate paired samples were analyzed on Day 0; representative histograms are shown. Note that fluorescence intensity was 1400 times greater in the labeled group compared to the unlabeled group.

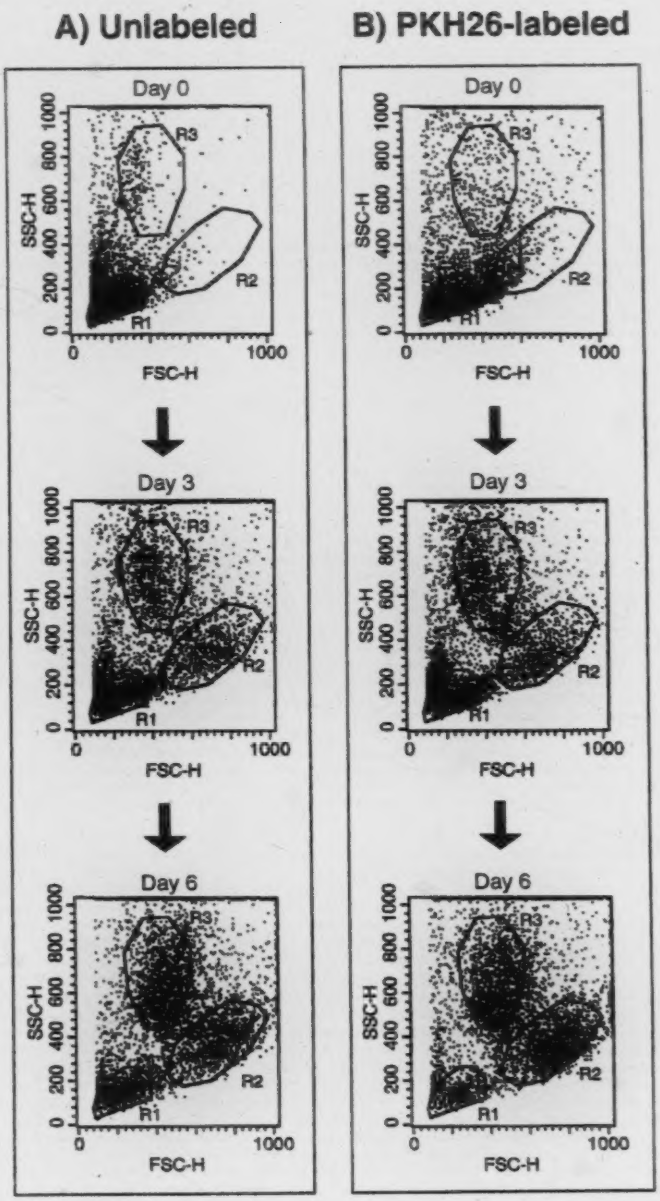

Figure 4.6: Flow cytometric analysis of PKM culture development in the absence (A) or presence (B) of PKH26. Cultures were established by seeding 1×10^7 kidney leukocytes into $25 cm^2$ tissue culture flasks in the presence of 25% (v/v) macrophage cell conditioned medium (CCM). Flow cytometric analyses were performed at various time points of cultivation. Triplicate cultures were established; the results of a representative replicate are shown.

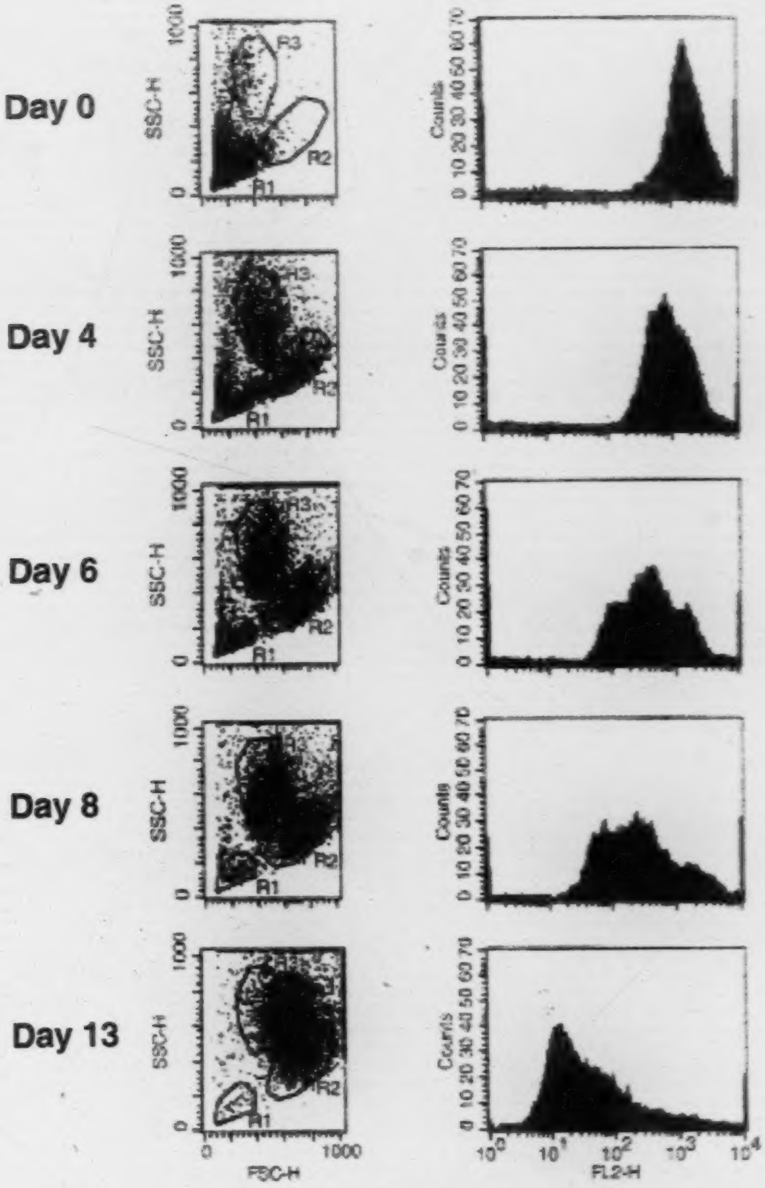

Figure 4.7: Proliferation analysis for developing primary kidney macrophages. 1×10^7 primary kidney leukocytes were isolated, labeled with PKH26, and cultured in $25 cm^2$ flasks in the presence of 25% (v/v) PKM cell conditioned medium. Fluorescence intensity was plotted on a four decade logarithmic scale. The fluorescence intensity over a thirteen day period was monitored. 10^4 events were analyzed at each time point. Triplicate cultures were analyzed; results for a representative replicate are shown.

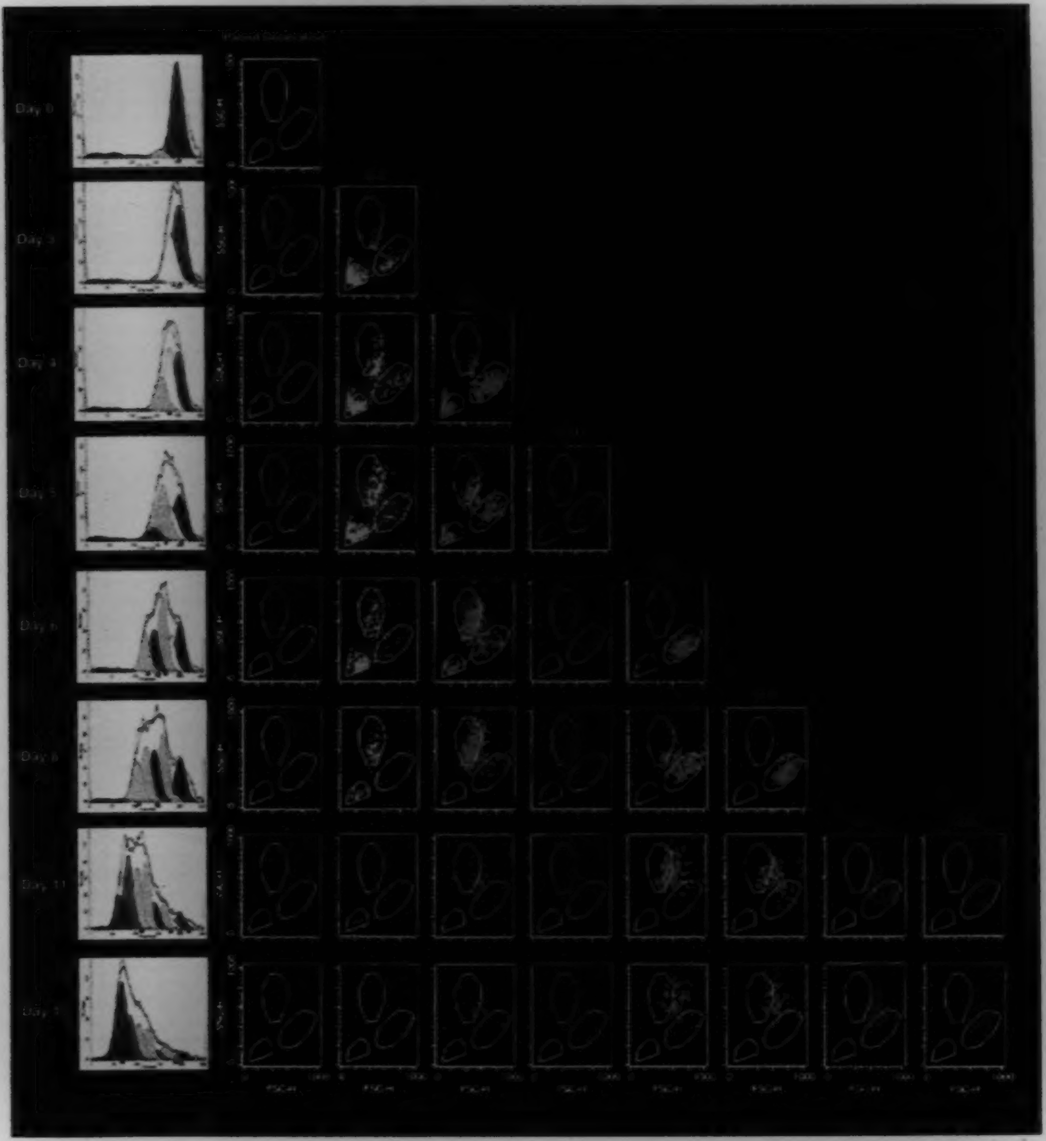

Figure 4.8: Flow cytometric analysis of proliferation and differentiation events during macrophage development *in vitro*. ModFit deconvolution of single log integrated fluorescence (FL2-H) histograms determined the number of generations that appeared during cultivation. The relative position of cells in each generation within FSC-H vs. SSC-H plots was determined using FACS analysis of ModFit deconvoluted histograms. Analyses were done on triplicate samples; results for a representative replicate are shown.

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CHAPTER V: CHARACTERIZATION OF DIFFERENT PHASES OF GOLDFISH MACROPHAGE DEVELOPMENT *IN VITRO*[4]

1. INTRODUCTION

Macrophage development is essential to the establishment of a properly working immune system and the regulation of homeostasis in the host (1-5). Colony stimulating factors including IL-3, GM-CSF, and CSF-1, as well as a variety of other cytokines (e.g. IL-4, IL-13) induce up-regulation of macrophage production (6-10). These modulators of macrophage hematopoiesis, however, do not work alone. They are part of a well-orchestrated network of intrinsic and extrinsic regulators that together guide a pluripotent stem cell to develop along the macrophage lineage. It is the intricate regulation of this hematopoietic process that leads to the generation of mature effector cells that are tailored to the immunological demands and the environment where they reside.

Equally important to the induction of macrophage development are the mechanisms by which cytokine signals can be down-regulated. A delicate balance exists between cytokine production, consumption, and inhibition during the regulation of macrophage hematopoiesis. The interruption of any of the pathways leading to up-regulation of the hemopoietic machinery ensures that the powerful effects of growth factors do not linger when the inductive stimuli have subsided (11). This is a central feature during the normal physiological turnover of cells, required to maintain the immunological readiness-state needed for optimal host defense.

Apoptosis provides a mechanism by which cellular turnover can take place without the induction of an inflammatory response. Kerr and colleagues (12) were the first to describe the distinct morphological changes that occur in apoptosis during normal tissue homeostasis. Subsequent studies have demonstrated that apoptosis represents a form of cellular suicide initiated through the activation of an internally encoded and evolutionarily conserved program (13). The physiological control of apoptosis provides a mechanism for the elimination of cells that have been damaged, produced in excess, or developed improperly.

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[4] A version of this chapter has been published:

Barreda and Belosevic (2001) *Fish and Shellfish Immunology*. 11: 169-186.

D.R. Barreda was involved in the data collection, analysis, and writing of the manuscript.

The induction of macrophage apoptosis and normal tissue turnover is related to the constant supply of survival signals provided by neighboring cells, the extracellular matrix, or growth factors arriving from circulation (13). A decrease in growth factor activity commonly results in an acute perturbation in cellular metabolism and the induction of cell cycle arrest, followed by the activation of an apoptotic response. Alternatively, growth factor withdrawal may result in the activation of a stress response that culminates in sensitization through cell death receptors (13).

A similar scenario appears to be present in fish macrophage hemopoietic systems. In Chapter III, I demonstrated that a goldfish (*Carassius auratus* L.) macrophage cell line (GMCL) and primary kidney macrophages (PKM) produce endogenous macrophage growth factor(s) (MGF). These macrophage growth factors accumulate in cell culture supernatants (CCM) and can be used to induce further proliferation of macrophages. Flow cytometric analysis of PKM, labeled using the fluorescent lipid membrane-bound dye, PKH26, allowed the examination of the proliferation potential and the differentiation pathways involved in PKM development (Chapter IV). Curiously, our results indicated that PKM entered a period of senescence following CCM-induced proliferation. Our working hypothesis was that the cessation of PKM proliferation was due to a decrease in growth-promoting activity of the collected CCM as PKM cultures entered a senescent state. In this chapter, I report on the growth-inducing activities of CCM isolated from different phases of PKM development. Moreover, a link was identified between the seeding-CCM composition, the extent of PKM growth, and an increase in apoptosis of PKM as they enter into a senescent state.

2. RESULTS

Although developing primary kidney macrophages (PKM) readily secrete MGF into culture supernatants, preliminary observations of the growth-inducing capabilities of various CCM batches indicated significant heterogeneity, even when all CCM were derived from Day 8 cultures (Fig. 5.1). In addition to overall cell growth, there was a correlation between the growth-inducing activity of CCM seeded and the kinetics of growth of the resulting PKM cultures. Morphological and flow cytometric analysis of the PKM cultures from which the CCM were collected indicated that those cultures yielding CCM with poor MGF activity had decreased proliferation, and possessed characteristics of cultures entering a "*senescence*" phase: cellular clumping, increased amount of adherent and giant multinucleated cells, and increased cellular vacuolization were some of the early features observed, and this was followed by cell death and debris formation at later stages. In contrast, PKM cultures yielding CCM with high MGF activity had morphological and flow cytometric characteristics typical of PKM cultures at the peak of their "*proliferative*" phase.

We hypothesized that PKM development was not merely time dependent. Instead, we believed that various microenvironmental conditions, which affected the rate of development of macrophage progenitors in response to exogenous MGF, stemmed from the physiological state of the donor fish. In addition, we reasoned that PKM cultures secreted a different spectrum of factors depending on their developmental state. Thus, a closer morphological and flow cytometric characterization of PKM cultures would result in a more accurate representation of the developmental phases of PKM.

2.1. Phases of PKM development

Detailed observation of the development of PKM cultures indicated that macrophage growth can be divided into three developmental phases, defined using morphological and flow cytometric analyses: a *lag* phase, a *proliferative* phase, and a *senescence* phase (Fig. 5.2). The *lag* phase represented the initial period of PKM development following the isolation of kidney leukocytes from goldfish hemopoietic tissues. Typically, this phase lasted 3-5 days. It was characterized by an initial sharp decrease in cell numbers and debris formation as cells outside the macrophage lineage (e.g. granulocytes, lymphocytes, NK cells) died off, presumably due to the absence of specific growth factors. Some proliferation and differentiation occurred, as early macrophage progenitors migrated toward the monocyte and macrophage flow cytometry-denoted gates (Fig. 5.2A); during this phase, 2-3 generations were detected by PKH26 analysis (Chapter IV), however, overall cell number in both non-adherent and adherent populations remained low. The end of the *lag* phase was characterized by a considerable decrease in the amount of debris, a decrease in vacuolization of the adherent macrophage population, and the percentage of putative monocytes approached 12% as determined by flow cytometric analysis (Fig. 5.2AB). The *proliferative* phase of PKM development represented the period in which most of the proliferation (typically 6 to 8 generations) and differentiation occurred, as determined by PKH26 analysis (Chapter IV). At the peak of the *proliferative* phase: a) three subpopulations of cells were identified in PKM cultures; b) no debris or cellular clumps were evident; and c) an established adherent population, and a high number of "healthy" non-adherent cells were observed (Fig. 5.2AB). The start of the *senescence* phase of PKM development was marked by the shift of the monocyte subset, followed by the mature macrophage subset, out of the flow cytometry-defined gates towards a greater FSC-H (Fig. 5.2A). This was followed by cessation of proliferation events, cellular vacuolization, appearance of multinucleated giant cells, cellular clumping, increased debris formation, and ultimately cell death (Fig. 5.2B).

2.2. Detection of apoptosis in the *senescence* phase of PKM cultures

Microscopic examination of PKM cultures in the *senescence* phase of PKM development indicated the presence of several cells in the late stages of apoptotic cell death (Fig. 5.2B). To further examine this possibility, genomic DNA isolated from representative macrophage cultures in the *proliferative, early senescence, and late senescence* phases were analyzed using the DNA fragmentation assay (Fig. 5.3). DNA fragmentation yielded a characteristic "ladder" indicating nucleosomal fragment formation in the *senescence* phase DNA samples (14-16). The DNA ladder was detected even when 0.5 μg of genomic PKM *senescence* phase DNA was subjected to gel electrophoresis. In contrast, even when as much as 1.5 μg of *proliferative* phase genomic DNA was electrophoresed, no DNA fragmentation was evident. These results suggest the absence of detectable levels of apoptosis in DNA samples prior to entrance of PKM cultures into the *senescence* phase of development.

2.3. Characterization of MGF activity in CCM at different phases of PKM development

The growth-inducing activities of PKM supernatants were quantitated based on time (Fig. 5.4A) and PKM developmental phase (Fig. 5.4B). Figure 5.4A shows the variability that exists between the time at which maximum MGF activity was observed in supernatants from four PKM cultures. In contrast, characterizing PKM supernatants based on developmental phase normalized MGF activity: maximum activity was consistently found in supernatants isolated from PKM cultures during the *proliferative* phase of development (Fig. 5.4B).

Addition of *proliferative* phase CCM consistently resulted in higher cellular yields after 8 days of cultivation, as described above. We also tested the *proliferative* phase CCM's ability, compared to other phases CCM, to extend the *proliferative* phase of subsequent PKM cultures. The low seeding inoculum (8.5×10^6 cells per $25 cm^2$ culture flask) was chosen to minimize the effect of endogenous MGF production, thus maximizing MGF effect from added CCM samples. As shown in Fig. 5.5 PKM cultures seeded with *proliferative* phase CCM, particularly at late stages, remained in the *proliferative* phase for a longer period of time compared to parallel cultures seeded with CCM from other phases. Furthermore, addition of increasing amounts of *proliferative* phase CCM led to improved culture growth and a prolonged *proliferative* phase in subsequent PKM cultures (Fig. 5.6). This was consistent with our previous results that showed that the amount of PKM growth was directly proportional to the amount of exogenous MGF added (Chapter III). A link between the amount of CCM seeded and the rate of entrance of PKM cultures into the *senescence* phase was also established. Further experiments have demonstrated that proliferating PKM cultures can be maintained for several months, provided that appropriate amounts of CCM, containing high MGF activity, were supplied to the cultures (data not shown). Overall, collection of PKM cell conditioned supernatants based on developmental phase rather than time has led to the generation of PKM cultures that consistently provided higher cellular yields, as well as CCM with higher MGF activity. This facilitated future experiments that required high cell numbers, as well as our efforts to purify the native MGF.

2.4. Alteration of macrophage development by *senescence* phase CCM

Analysis of PKM development in response to *senescence* phase CCM showed that it was a poor inducer of macrophage growth (Fig. 5.4B). Even in cases where PKM developed in the absence of exogenous MGF, parallel PKM cultures seeded with *senescence* phase CCM developed poorly (Table 5.1). It does not appear that the lack of PKM growth was due to over-stimulation by high MGF loads since a decrease in the concentration of the *senescence* phase-derived CCM still resulted in poor PKM growth (Fig. 5.7). Moreover, CCM with high MGF activity, concentrated up to 20x, has been observed to yield significant macrophage growth (data not shown). Cultures established in the presence of *senescence* phase CCM were characterized by failure to develop into the putative monocyte and macrophage subsets (no migration to the corresponding flow cytometry-denoted gates), poor culture morphology, and increased debris formation. Interestingly, addition of *senescence* phase CCM to equal amounts of *proliferative* phase CCM completely abrogated the growth-inducing capacity of the cell conditioned supernatants (Fig. 5.8). Taken together these results suggest the presence of proliferation inhibitory signals in *senescence* phase CCM.

3. DISCUSSION

I previously described the secretion of macrophage growth factor(s) (MGF) into culture supernatants by a goldfish macrophage cell line (GMCL) and primary kidney macrophages (PKM) (Chapter III). Analysis of the development of PKM cultures in response to these CCM, however, indicated a high degree of variability between cultures from individual fish, both in the rate of development and in the growth-inducing capabilities of the CCM. Since the initial primary cultures were obtained from an outbred population of fish, one would expect a marked difference in the immunological competence of individual fish due to a variety of factors including age, sexual maturation, and changes in hormonal levels due to seasonal variations (17, 18). We hypothesized that this heterogeneity could be minimized significantly by taking a closer look at PKM development. In this chapter, I report that PKM growth can be subdivided into developmental phases, defined using both morphological and flow cytometric characteristics. Moreover, the magnitude of MGF activity was related to a particular developmental phase, allowing for normalization of MGF activity and facilitating more precise characterization of MGF.

The regulation of tissue cell number is a critical homeostatic objective (19-23). A delicate balance between the processes of proliferation, differentiation, and cell death must be attained to ensure the maintenance of immune competence required for optimal host defense. During normal tissue turnover, apoptosis provides an evolutionary conserved mechanism that allows for the removal of excess or expired cells (24-27). The apoptotic events observed as PKM cultures enter the *senescence* phase of development may represent a control mechanism fundamental to the conservation of homeostasis. Apoptosis is internally directed; cellular components are dismantled within the confines of an intact cell membrane, and rapid ingestion by phagocytic cells prevents local inflammation (28). In mammals, stem cell renewal is continuously counter-balanced by apoptosis of functionally inactive or terminally differentiated cells. This extensive cellular turnover, demanded by functional aspects of the hemopoietic system, provides a flexible mechanism for dictating the cellular composition of the mature population and for controlling population size (28).

Although a number of mechanisms have been proposed for the initiation of the apoptosis cascade a common theme arises: the removal of survival signals, such as those derived from cytokine growth factors, results in the shift of the life-death equilibrium activating cell deletion mechanisms. For example, M-CSF and other colony stimulating factors have been shown to enhance macrophage survival by preventing the induction of an apoptotic program (2, 7, 13, 29, 30). Conversely, removal of the growth factor signals results in triggering of the apoptotic cascade and cell death (13, 30-32). Similarly, we have found that MGF concentration was a key element in determining whether a PKM culture remain in the *proliferative* phase or enter a *senescence* phase characterized by cessation of proliferation and apoptotic cell death. It is, therefore, not surprising that the establishment of the optimal conditions has led to the long-term maintenance of a number of PKM cultures in our lab, and the generation of several fish macrophage cell lines (33-38).

A number of mechanisms, geared towards the removal or inactivation of growth-inducing signals, have been described in mammalian systems (11, 39, 40). Several positive and negative regulators of macrophage proliferation, produced in response to a variety of local and systemic environmental factors (including cell density, nutrient deprivation, cell size, functional demands of the immune system), have been implicated in the maintenance of hemopoietic homeostasis (2, 28, 41). These factors maintain the necessary balance between proliferation and cell death and are required for cellular turnover. Thus, the production of proliferation inhibitory signals leading to apoptotic PKM death during the *senescence* phase of development represents an attractive model for the regulation of macrophage numbers during monocytopoiesis in fish. Moreover, the identification of the apoptotic process as part of the normal development of fish macrophages presents some key considerations: a) it suggested the importance of apoptosis as a control mechanism of fish macrophage development; b) it demonstrated that this control was intrinsic to cells of the macrophage lineage; and c) it provided a unique opportunity for the study of apoptotic regulatory mechanisms in teleost macrophages in relation to a specific phase of *in vitro* development.

There are also important practical aspects to the findings reported in this chapter. Isolation of PKM cell conditioned media at the peak of macrophage growth maximized MGF activity in the CCM and prevented the introduction of negative regulators of PKM proliferation, thus contributing significantly to our MGF purification and characterization efforts. The characterization of teleost macrophage growth factors will be the first major step in understanding not only the physiology of fish macrophages but also hematopoiesis.

It is well established for mammalian macrophages that initial cytokine exposure determines macrophage functional status and in many cases renders them unresponsive to other cytokines that regulate opposing functions (42). The use of PKM at the peak of their growth, prior to the generation of apoptosis-inducing signals, will allow for the selection of specific macrophage subsets for antimicrobial research projects. For example, it will enhance the collection of PKM that are responsive to activation / deactivation signals rather than those which may already be committed to the apoptotic pathways.

Table 5.1: Growth of primary kidney macrophages (PKM) in response to supernatants collected at the different phases of development.[a]

Phase at which seeding CCM was collected	Percent of developing PKM cultures[b]	Length of Proliferative Phase (Days ± SEM)[d]	Percent of cultures entering the Senescence Phase[e,f]
Negative Control (No CCM added at Day 0)	60.9% (14/23)	2.4 ± 0.4	64.3% (9/14)
Lag Phase	100% (6/6)	3.7 ± 0.9	66.7% (4/6)
Proliferative Phase-early	100% (13/13)	4.2 ± 0.9	38.5% (5/13)
Proliferative Phase-late	100% (60/60)	4.9 ± 0.8	21.7% (13/60)
Senescence Phase	6.3% (1/16)[c]	1	100% (1/1)

[a] PKM cultures were established by seeding 8.5×10^6 kidney leukocytes per $25 cm^2$ culture flasks in the absence or presence of supernatants from the various phases of PKM development. The kinetics of growth of the developing cultures was determined by morphological and flow cytometric analysis. A developing culture was defined as one that reached the *proliferative* phase of development.

[b] The ratio denotes the number of PKM cultures that developed versus the total number of seeded cultures.

[c] Of 16 cultures seeded with *senescence* phase CCM 15 failed to develop. In 4 of these 15 cases PKM cultures failed to develop even though parallel cultures seeded with no CCM reached the *proliferative* phase. Moreover, note that only 1/16 of the PKM cultures in this group reached the *proliferative* phase, and that this was short-lived (1 day) and quickly followed by entrance into the *senescence* phase.

[d] Values were calculated based on the number of developing macrophage cultures in the corresponding group. Greater values indicate a longer period of PKM proliferation, which translates to a delayed entrance into the *senescence* phase.

[e] The ratio denotes the number of PKM cultures that entered the *senescence* phase versus the total number of developing cultures in the corresponding group.

[f] Values are based on an eight day observation period.

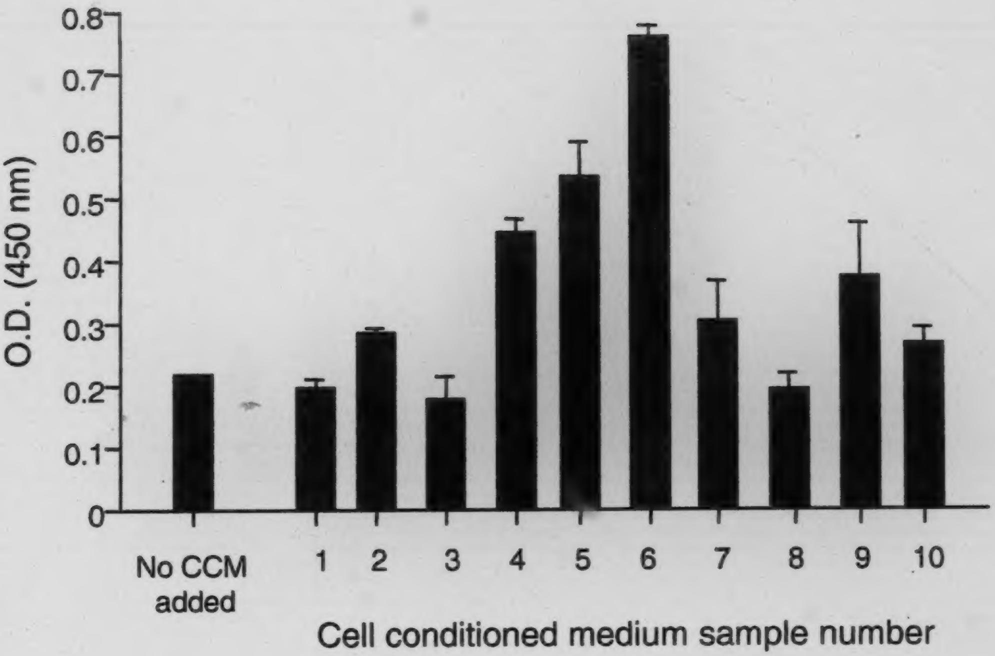
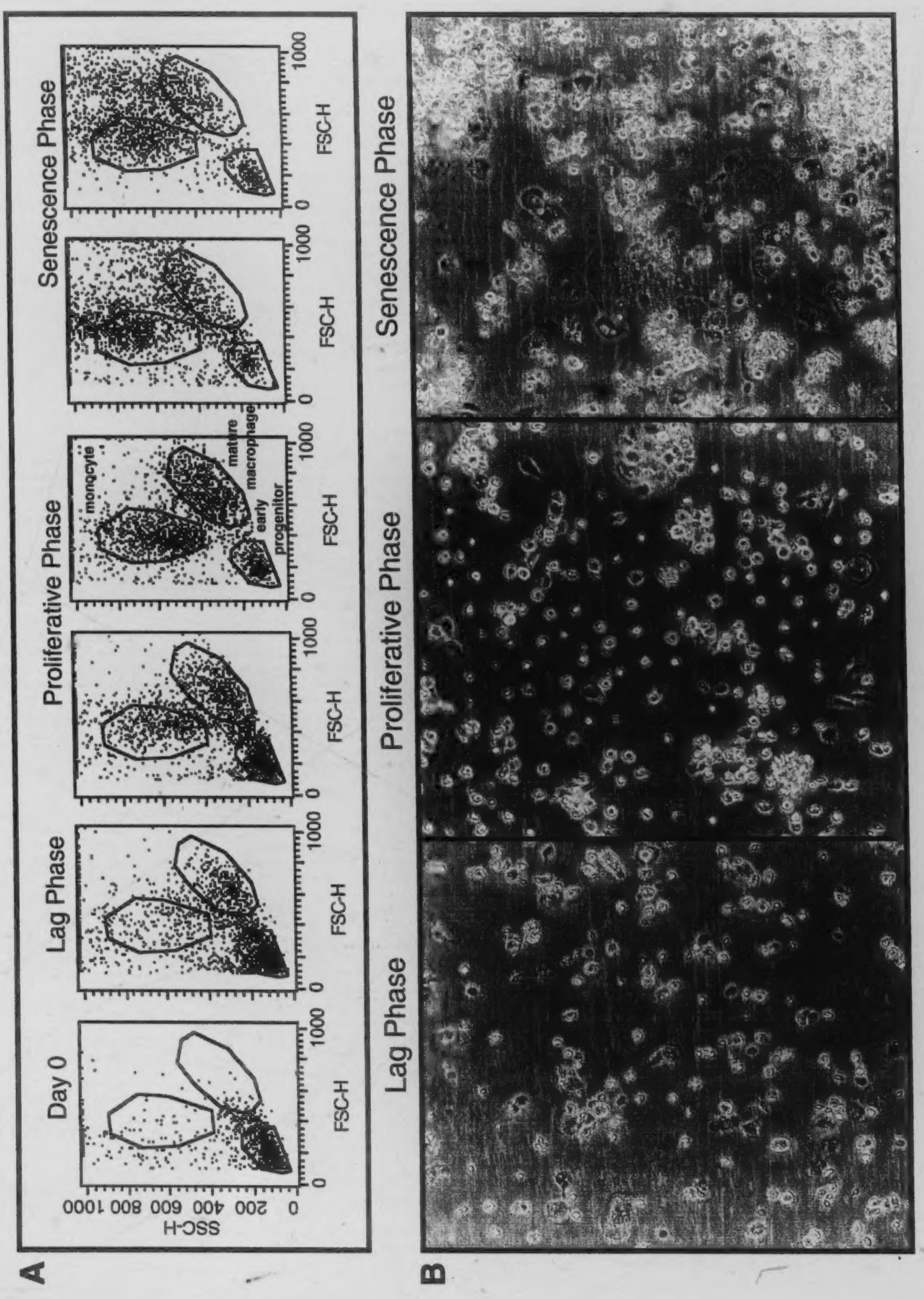


Figure 5.1: Heterogeneity in the growth-inducing activities among cell conditioned media from representative PKM cultures. Supernatants from typical Day 8 PKM cultures were collected and tested for their ability to induce proliferation of kidney leukocytes in microtitre cultures seeded with 2.5×10^4 cells per well in 96-well culture plates. Cell numbers were indirectly determined using the XTT tetrazolium assay after 8 days of cultivation. Each data point represents the mean O.D. ± SEM of triplicate wells. The experiment was repeated on kidney leukocytes isolated from three fish; representative results are shown.

Figure 5.2: Phases of primary kidney macrophage development. (A) Flow cytometric analysis of the kinetics of PKM development. Note the progression from a culture composed mainly of early macrophage progenitors at Day 0, to one composed of three macrophage subpopulations (early progenitors, monocytes, and mature macrophages) at the peak of the *proliferative phase*, to one entering a *senescence phase* characterized by cell death and aggregate formation that results in an increase in the average size of analyzed events (greater FSC-H). (B) Representative phase contrast micrographs of PKM cultures in *Lag*, *Proliferative*, and *Senescence phases* of development. Note distinguishing factors such as overall cell numbers (adherent and non-adherent populations), cell morphology, cellular vacuolization, formation of "cell islands", presence of debris, cellular clumping, and cell death (typical examples of cells in the late stages of apoptosis are shown with black arrows).


A
Day 0
Lag Phase
Proliferative Phase
Proliferative Phase
Senescence Phase
Senescence Phase
SSC-H
0
200
400
600
800
1000
FSC-H
monocyte
mature macrophage
early progenitor
B
Lag Phase
Proliferative Phase
Senescence Phase

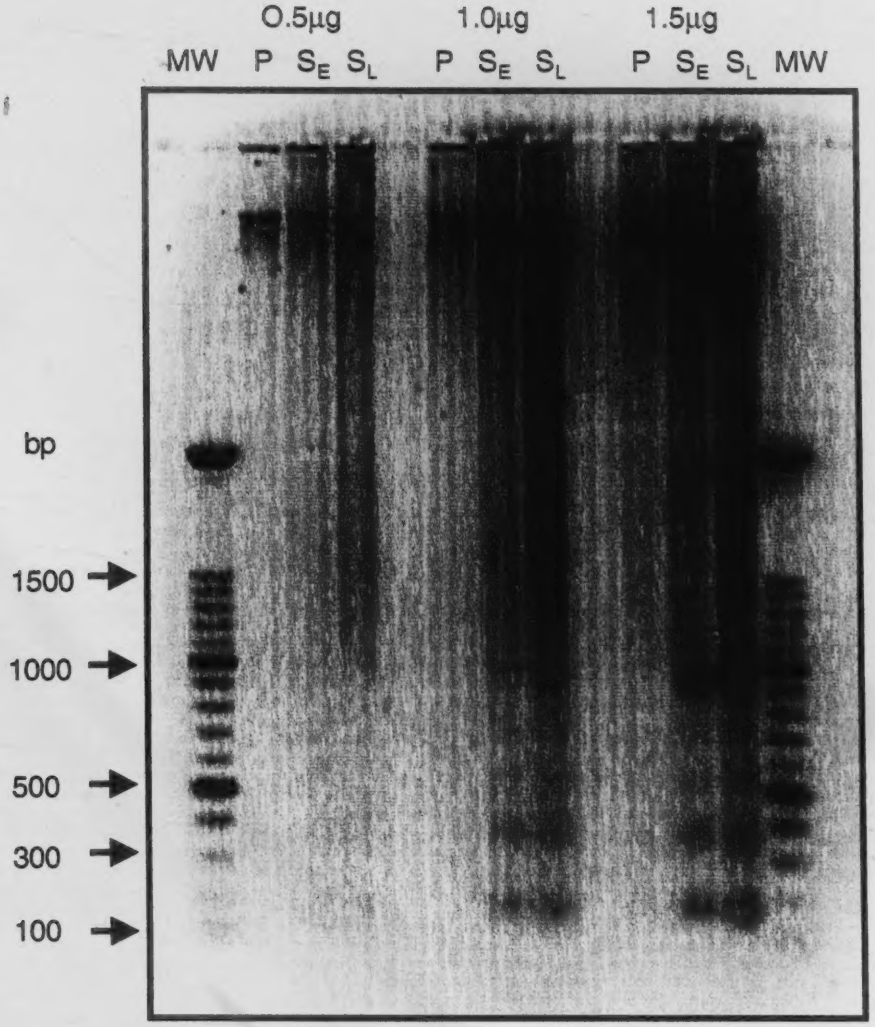

0.5μg
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Figure 5.3: Detection of apoptotic cell death in primary kidney macrophage cultures. Genomic DNA isolated from *proliferative* (P), *early senescence* (S_E), and *late senescence* (S_L) *phases* of PKM development was analyzed using the DNA fragmentation assay. Triplicate samples were collected for each phase; representative samples are shown. 0.5μg, 1.0μg, and 1.5μg of genomic DNA were loaded for each sample. Nucleosomal ladders were visualized using ultraviolet light following EtBr staining of the 0.8% agarose gel. MW- 100bp DNA size markers.

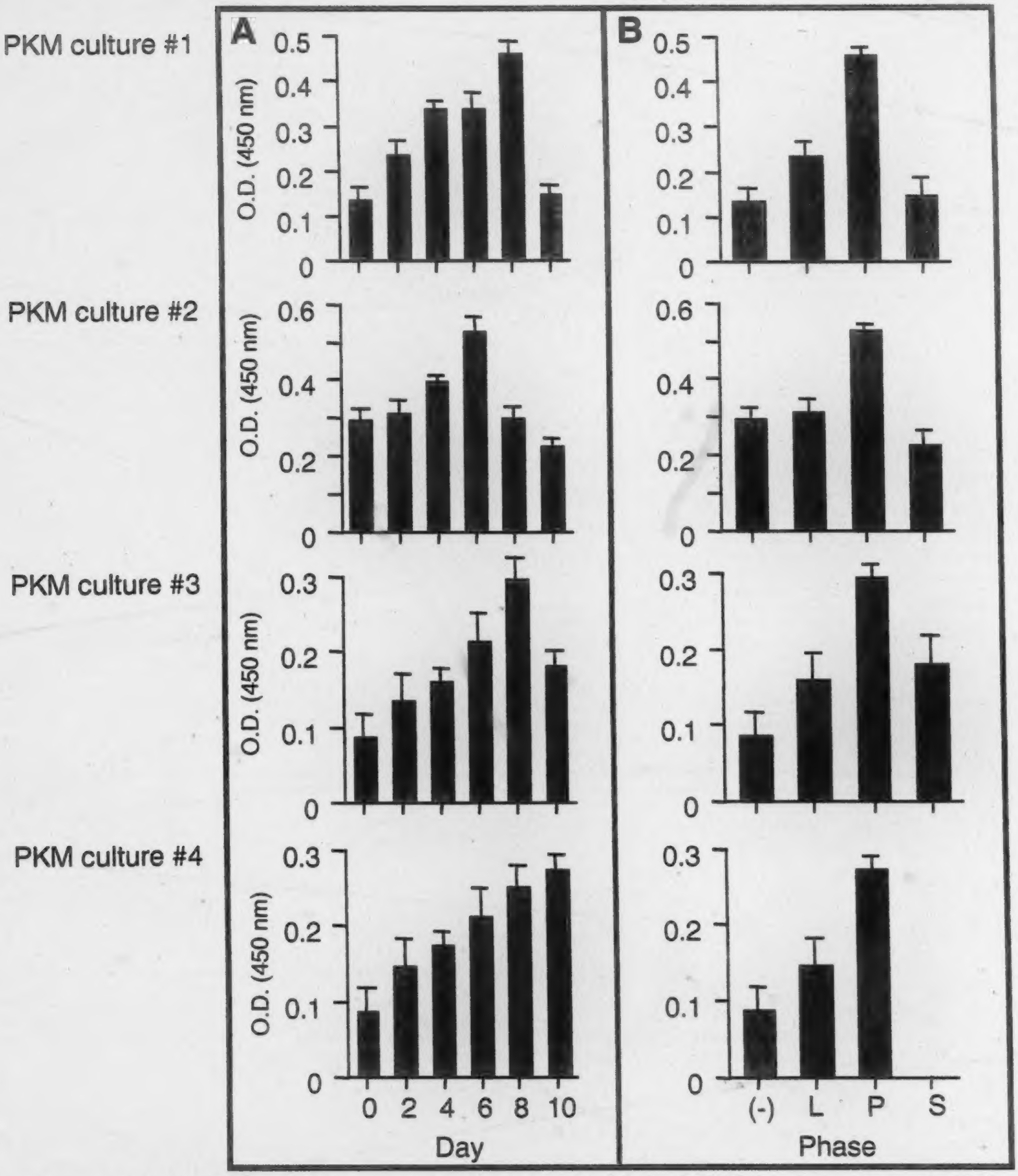

Figure 5.4: Analysis of the growth-inducing activities of PKM supernatants based on Time (A) or Phase of development (B). Supernatants from four PKM cultures were collected at 2-day intervals during PKM development and tested for their ability to induce proliferation of kidney leukocytes in microtitre cultures seeded with 2.5×10^4 cells per well in 96-well culture plates. Cell numbers were indirectly determined using the XTT tetrazolium assay after 8 days of cultivation. Each data point represents the mean O.D. ± SEM of triplicate wells. Note the accurate correlation of CCM growth-inducing activity between the various cultures upon characterization based on developmental phase rather than time.

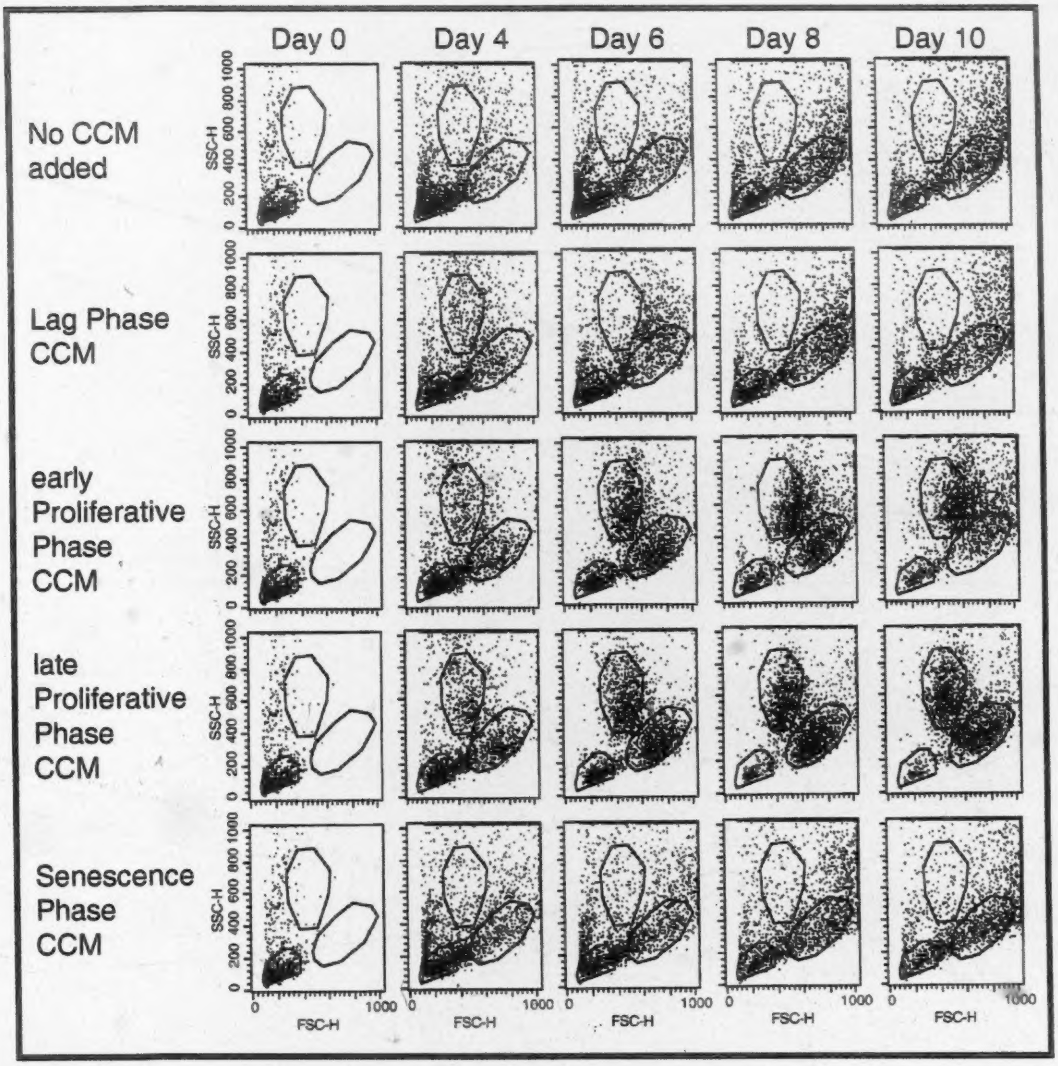

Figure 5.5: Effect of PKM phase of development on the growth-inducing activities of CCM. Supernatants from PKM cultures at the different phases of PKM development were collected and tested for their ability to induce subsequent PKM culture growth. The kinetics of PKM growth were observed at five intervals during a 10 day observation period. Only those groups seeded with *lag*, early and late *proliferative phase*-derived CCM underwent normal PKM development, passing through *lag*, *proliferative*, and *senescence phases*. Furthermore, note the increasing length of time that cultures remain in the *proliferative phase* in these three groups, reaching a peak when late *proliferative phase* PKM CCM was used. In contrast to the latter group, where the resulting PKM culture failed to enter the *senescent phase*, cultures seeded with *senescence phase*-derived CCM or No CCM failed to develop. The experiment was repeated on kidney leukocytes isolated from 7 fish; representative results from one fish are shown.

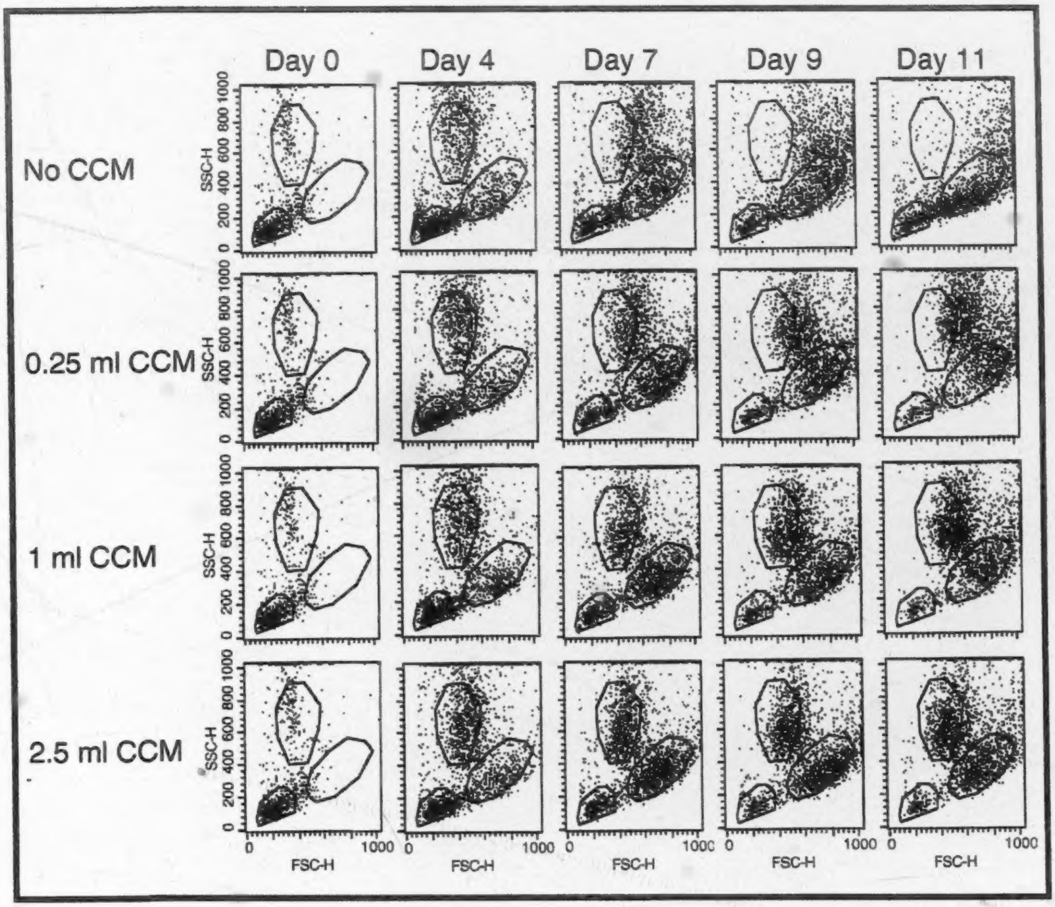

Figure 5.6: Effect of MGF concentration on the kinetics of primary kidney macrophage growth. Varying amounts of *proliferative phase* CCM were used to seed PKM cultures. The kinetics of PKM growth were observed at five intervals during an 11 day observation period. All groups underwent normal PKM development, passing through *lag*, *proliferative*, and *senescence phases.* However, note the increasing length of time that cultures remain in the *proliferative phase* in those groups seeded with higher amounts of exogenous MGF. Conversely, the rate of entrance into the *senescence phase* was faster in those groups seeded with lower amounts of exogenous MGF; when 2.5ml of CCM was used the resulting PKM culture failed to enter the *senescence phase* during the observation period. The experiment was repeated on kidney leukocytes isolated from 6 fish; representative results from one fish are shown.

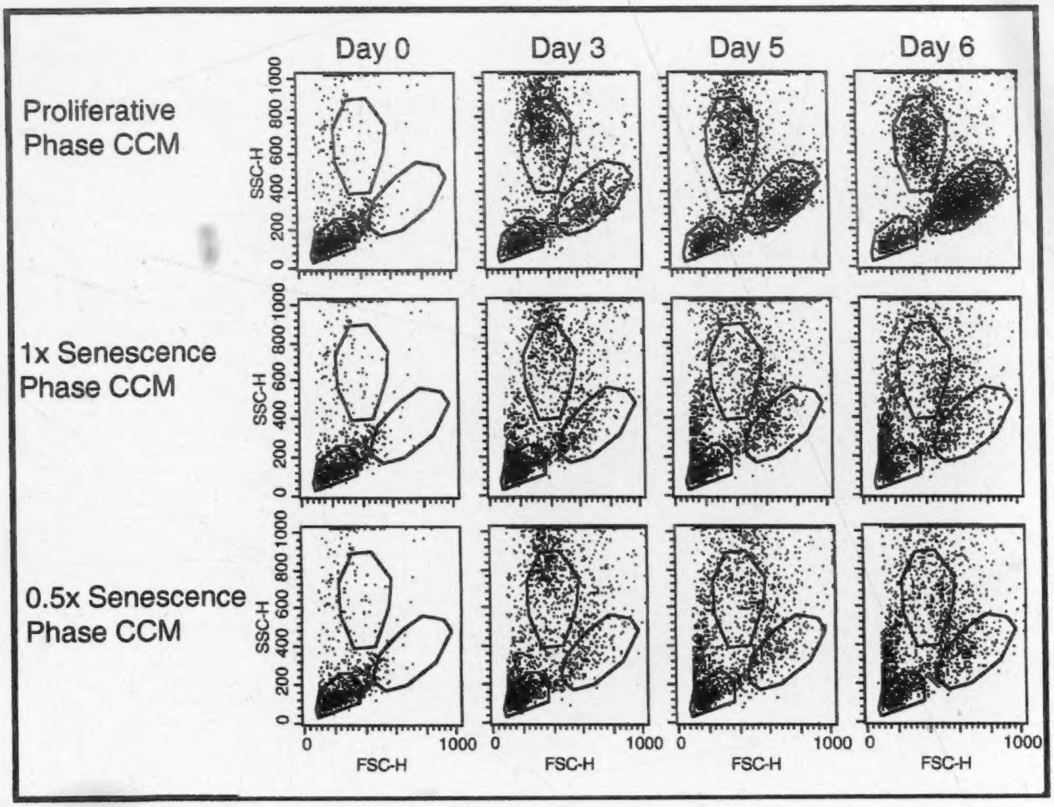

Figure 5.7: Inhibition of primary kidney macrophage growth in response to *senescence phase* supernatants is not a result of over-stimulation due to high MGF concentration. Supernatants from *senescence phase* PKM cultures at different concentrations were tested for their ability to induce PKM growth. The kinetics of PKM growth in response to each stimulus was observed in cells isolated from three fish; flow cytometric analyses of PKM development for a representative fish are shown.

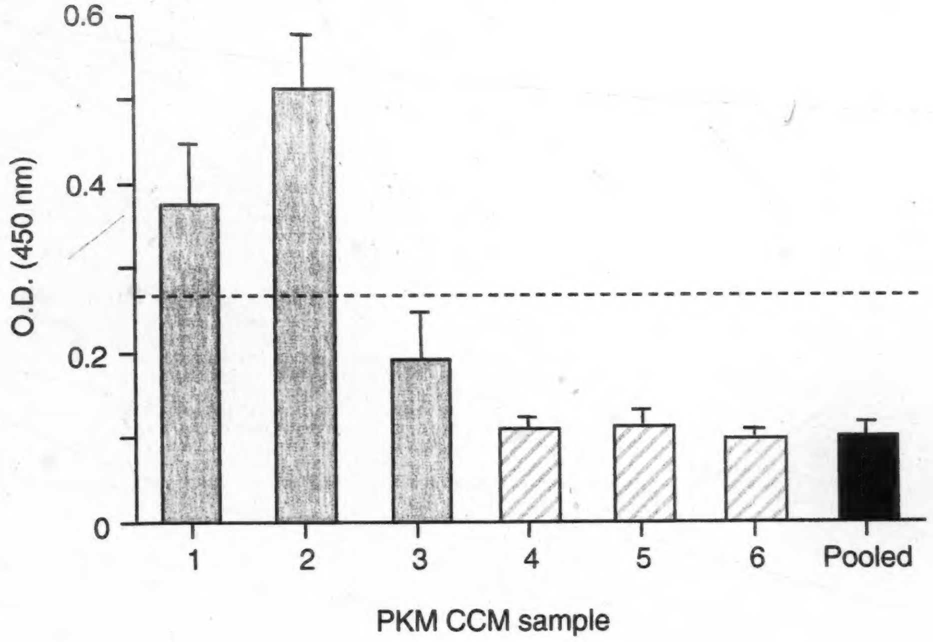

Figure 5.8: Identification of putative macrophage proliferation inhibitory signals in PKM culture CCM. Supernatants derived from six PKM cultures in the *proliferative* (grey bars) and *senescence phase* (hatched bars) of PKM development possessing high and low growth-inducing capabilities respectively were combined in equal amounts (black bar). All CCM samples were then tested for their ability to induce proliferation of kidney leukocytes in microtitre cultures seeded with 2.5×10^4 cells/well in 96-well culture plates. Cell numbers were indirectly determined using the XTT tetrazolium assay after eight days of cultivation. Each data point represents the mean O.D. ± SEM of triplicate wells. The dashed line represents the expected growth-inducing activity of the pooled sample if growth-inhibitory activity in senescence phase supernatants was absent.

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CHAPTER VI:
NEGATIVE CONTROL OF FISH MACROPHAGE HEMATOPOIESIS BY A NOVEL SOLUBLE FORM OF THE CSF-1 RECEPTOR [5]

1. INTRODUCTION

The colony-stimulating factors (CSFs) are a group of cytokines central to the development of blood cells, the modulation of their functional responses, as well as the maintenance of homeostasis and overall immune competence (1). Among the CSFs, the macrophage colony-stimulating factor or CSF-1 is the principal regulator of the survival, proliferation, and differentiation of macrophages and their precursors (2, 3). CSF-1 also synergizes with other cytokines to mediate the proliferation of early hematopoietic progenitors (4-6). In the mammalian system, macrophage progenitors depend on a variety of cell types within the surrounding microenvironment for the production of CSF-1 and its subsequent induction of hematopoietic events. The biological effects of CSF-1 are mediated via its cognate receptor (CSF-1R), a large integral plasma membrane glycoprotein encoded by the c-*fms* gene (2, 3). The selective expression of the CSF-1R on the surface of macrophages parallels their responsiveness to CSF-1, increasing progressively as early progenitors advance through the macrophage differentiation pathway. This feature has made the CSF-1R a useful marker of macrophage differentiation. In fact, the c-*fms* gene represents the most universal marker known so far, both across the heterogeneous macrophage populations of humans and mice, as well as across species, from fish to humans (7-10).

Similarly to the other colony-stimulating factors, CSF-1 production and degradation is strictly controlled, thus allowing for effective modulation of its biological functions in steady-state conditions as well as under periods of stress. Moreover, the mechanisms behind its expression and that of its cognate receptor ensures that CSF-1-

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[5] A version of this chapter will be submitted for publication in 2003:
Barreda, Hanington, and Belosevic.
D.R. Barreda was involved in the data collection, analysis, and writing of the manuscript.

mediated actions are tightly coordinated, within the context of a network of complex but finely tuned regulatory pathways derived from a variety of local and endocrine hematopoietic regulators. In addition to mechanisms regulating gene expression of CSF-1 and its receptor, CSF-1 activity can be effectively controlled through metabolic processing, receptor-mediated endocytosis, or by direct inhibition of the signaling pathways triggered by receptor-ligand interactions (discussed in detail in Chapter I). Preliminary analysis of the endogenous macrophage growth factor (MGF) in primary kidney macrophage (PKM) cultures, as well as characterization of phenotypic and gene expression changes that occur as PKM develop, suggested the presence of a CSF-1-like molecule in goldfish macrophage culture supernatants. Our attempts to purify the CSF-1-like molecule from PKM cell-conditioned media (CCM), however, were hindered by the very low concentrations at which it appeared to exert its strongest biological activity. We therefore decided to isolate the corresponding receptor as a viable alternative for the subsequent purification and biochemical characterization of its native ligand.

In this chapter, I described our first steps in the characterization of the CSF-1/CSF-1R regulatory system in goldfish primary kidney macrophages. Two cDNA libraries, generated at different phases of macrophage development, were screened with cDNA probes directed against c-*fms*, the gene encoding for the CSF-1R. Surprisingly, during the screening procedure, I isolated a unique transcript that only encoded for the extracellular portion of the CSF-1R. This represented a full-length mRNA species as it contained a start codon, signal peptide, stop codon, polyadenylation signals, and a poly-A tail. This transcript was only identified in the *senescence* phase cDNA library, which corresponded to the period in PKM development characterized by cessation of proliferation events, cellular clumping, debris formation, and ultimately cellular death through a process of apoptosis (Chapter V). Therefore, we hypothesized that it encoded for a novel soluble form of the CSF-1R (sCSF-1R) that may act in a negative fashion to regulate macrophage numbers following up-regulation of hematopoietic events.

2. RESULTS

2.1. Identification, cloning, and sequence analysis of the goldfish CSF-1 receptor

As a first step in the characterization of the CSF-1/CSF-1R regulatory system in primary kidney macrophages (PKM) we sought to identify the gene encoding for the CSF-1R in goldfish (Fig. 6.1). Previous studies had identified a gene encoding for the CSF-1R in two other fish species, zebrafish and puffer fish (11, 12). Alignment of these nucleotide sequences with those of human (X03663), mouse (X06368), and cat (J03149) CSF-1R gene sequences led to the identification of conserved regions and subsequent construction of degenerate primers. These primers were then used in the generation of goldfish-specific intra-exon gene fragments, which were radiolabeled and used to screen two PKM cDNA libraries.

Analysis of 26,000 clones led to the isolation of 8 putative c-*fms* clones (Fig. 6.2). PKM inserts in each of these clones were subsequently PCR-amplified and analyzed in secondary and tertiary screening procedures. Following identification and isolation of single-transcript c-*fms*-encoding clones (Fig. 6.3), 5' and 3'-rapid amplification of cDNA ends (RACE) led to the identification of the complete transcript encoding the full-length membrane-bound CSF-1R (mCSF-1R) in goldfish. The 2925 nucleotide open reading frame (ORF) encodes for a 975 amino acid polypeptide. Alignment of the deduced amino acid sequence for this transcript with those of zebrafish (*D. rerio*), puffer fish (*T. rubripes*), and human (*H. sapiens*) CSF-1R (Fig. 6.4) indicated a high degree of evolutionary conservation, particularly with respect to the functional structural components mediating ligand-binding and downstream signaling. The goldfish CSF-1R sequence was 86% identical and 93% similar to that of zebrafish. It was also highly homologous to trout (66% identical, 78% similar) and puffer fish (62% identical, 76% similar) CSF-1R sequences. Despite long evolutionary distances, goldfish CSF-1R also displayed significant homology to mammalian CSF-1Rs; the percent identities to human, rat, mouse, and cat were 44%, 44%, 43%, and 42%, respectively.

2.2. Identification of a unique CSF-1R transcript in a primary kidney macrophage (PKM) *senescence* phase cDNA library

In addition to identification of the goldfish membrane-bound CSF-1R (mCSF-1R), we identified a unique transcript encoding only for the extracellular ligand-binding portion of the CSF-1R. Sequence analysis revealed that it was derived from a full-length mRNA species containing a start codon, signal peptide, stop codon, polyadenylation signals, and a poly-A tail (Fig. 6.5). Alignment of its nucleotide sequence with that encoding goldfish mCSF-1R indicated that they were identical between the start codon and residue 619 (Fig. 6.6). Interestingly, this represented the putative exon4-intron4 boundary based on alignment with the human gene sequence.

This unique transcript was isolated from a PKM *senescence* phase cDNA library. Therefore, we hypothesized that this may represent a novel soluble form of the CSF-1 receptor that was secreted by primary kidney macrophages. We reasoned that this soluble CSF-1R (sCSF-1R) may inhibit CSF-1 activity through prevention of CSF-1 binding to its cognate receptor on the surface of culture macrophages. The decrease in CSF-1-mediated macrophage stimulation could, in turn, lead to the marked phenotypic changes that included down-regulation of proliferation and apoptotic cell death, which are characteristic of PKM cultures entering the *senescence* phase (Chapter V). Primary kidney macrophage cultures consist of three distinct subpopulations of cells, each corresponding to a distinct stage of macrophage development (early progenitors, monocytes, and mature macrophages). Determination of CSF-1R expression as part of a macroarray analysis of gene expression among the three stages of PKM development indicated that all three subpopulations contained CSF-1R transcripts (Fig. 6.7A). Subsequent analysis of transcript sequences allowed the construction of sCSF-1R-specific primers in order to determine which subpopulation(s) in PKM cultures was responsible for the production of sCSF-1R. Semi-quantitative RT-PCR analysis showed that the sCSF-1R transcript was readily detected in R1-early progenitors and R2-mature macrophages; in contrast, the sCSF-1R transcript was not detected in R3-monocytes (Fig. 6.7B). Interestingly, only the R1-early progenitors and R2-mature macrophage subpopulations have been shown to be capable of self-renewal in the absence of exogenous growth factors (Chapter III). Although preliminary, these results suggest that whereas membrane-bound CSF-1R was encoded by all three PKM subpopulations, the sCSF-1R expression may be restricted to the spontaneously growing R1-early progenitor and R2-mature macrophage PKM subsets. We are currently conducting further experiments to examine this at both mRNA and protein levels.

2.3. Expression of recombinant sCSF-1R and characterization of its biological activity

In order to determine whether the unique c-*fms* transcript encoded for a soluble form of the CSF-1R (sCSF-1R) that inhibited PKM development, we expressed this transcript in an insect-based protein expression system. The sCSF-1R transcript was engineered to contain a C-terminal 6xHis tag and a V5 epitope to facilitate subsequent purification and detection methodologies (Fig 6.8). The pIB/V5-His-TOPO sCSF-1R expression vector was transfected into *Drosophila* KC embryonic cells, as these would likely provide the necessary post-translational modifications to ensure biological activity. Analysis of stable transfectants determined that KC cells readily secreted recombinant sCSF-1R into culture supernatants and these supernatants inhibited the proliferation of PKM (Fig. 6.9 and 6.10). Subsequent purification of recombinant sCSF-1R confirmed that it could be effectively isolated by virtue of its 6xHis tag (Fig. 6.10B). Characterization of the effect of purified recombinant sCSF-1R treatment on PKM culture development indicated that it induced phenotypic changes associated with entrance into the *senescence* phase at nanomolar concentrations (Fig. 6.11). The faster rate of entrance into the *senescence* phase upon addition of recombinant sCSF-1R was reminiscent of PKM cultures treated with lower concentrations of MGF-containing supernatants (see Fig. 5.6, Chapter V). Moreover, PKH26 analysis of PKM proliferation following treatment with sCSF-1R revealed a clear inhibition of proliferation events at nanomolar concentrations (Fig. 6.12).

Imidazole derivates have been implicated in the inhibition of cellular proliferation, nucleic acid synthesis, inflammatory responses, and antigen presentation in a variety of cells including macrophages (13-15). However, imidazole is commonly used as a mild alternative for the elution of His-tagged proteins from nickel columns, particularly under native conditions. In order to ensure that any anti-proliferative effects of PKM were the result of sCSF-1R activity, we dialyzed purified recombinant protein fractions extensively against 1x D-PBS (2 x 4L changes of 1x D-PBS at 4°C over 48hr). Figure 6.13B shows how imidazole, even at concentrations significantly above those used in the elution protocol, did not have any significant effect on PKM proliferation. In contrast, a dose-dependent decrease in PKM proliferation was observed upon addition of recombinant sCSF-1R (Fig. 6.13A).

2.4. Production of an anti-sCSF-1R polyclonal antibody

We used purified recombinant sCSF-1R in the generation of an anti-sCSF-1R polyclonal antibody. As shown in Fig. 6.14A, this antibody effectively recognized recombinant sCSF-1R in a parallel western blot to that shown in Fig. 6.10B. However, the anti-sCSF-1R polyclonal antibody also recognized an additional protein band of approximately 75kDa, likely derived from insect proteins that were co-purified from KC cell supernatants.

Analysis of culture supernatants from PKM at different phases of development (Fig. 6.14B) indicated, most importantly, that sCSF-1R is produced as a soluble native protein by goldfish macrophages. Soluble CSF-1R concentration progressively increased in PKM supernatants from the *early proliferative* phase to the *senescence* phase. Interestingly, sCSF-1R could also be readily detected in serum containing medium and *lag* phase supernatants.

3. DISCUSSION

I previously described the production of endogenous macrophage growth factor(s) (MGF) by primary kidney macrophages (PKM) (Chapter III). This was an important observation as it pointed to fundamental differences between fish and mammalian macrophages. Whereas fish macrophages produce endogenous growth factor(s) to support their growth *in vitro*, descriptions of mammalian macrophage development indicated that they are dependent on a variety of cell types within the surrounding microenvironment for the production of growth factors like CSF-1, and the subsequent induction of hematopoietic events, particularly under steady-state conditions (16-18).

Analysis of sorted PKM subpopulations suggested that endogenous MGF production was limited to the early progenitor and mature macrophage subsets, as they did not require addition of exogenous growth factors to proliferate (Chapter III). It is interesting that only these two subpopulations appear capable of sCSF-1R production. One may hypothesize that this is a reflection of a negative regulatory loop designed to control the development of spontaneously growing macrophage populations, particularly if future experiments confirm the presence of a CSF-1-like molecule in PKM cell conditioned medium. I believe that as PKM enter the *proliferative* phase the concentration of MGF, containing CSF-1-like activity, increases in the culture supernatants and results in up-regulation of macrophage proliferation and differentiation events, characteristic of this phase of development. Various microenvironmental conditions (including cell density, nutrient deprivation, cell size) then lead to the activation of negative feedback loops and the production of negative regulators of proliferation (including sCSF-1R) geared to the maintenance of homeostasis/culture integrity. The concentration of sCSF-1R and perhaps other proliferation inhibitors then increases in culture supernatants, resulting in growth factor deprivation and entrance into the *senescence* phase, characterized by cessation of proliferation events, cellular clumping, debris formation, and cell death through a process of apoptosis. Although *in vivo* this process would be expected to result in the control of macrophage numbers and the maintenance of homeostasis, *in vitro* there is a limited availability of clearance mechanisms for soluble mediators and death products. As a result, senescent cultures are generally terminal unless their supernatants are replenished with fresh CCM, and *senescence* phase CCM was generally poorer at inducing PKM growth than even medium alone (Chapter V). Alternatively, the role of sCSF-1R *in vivo* may be quite distinct from that observed in our *in vitro* PKM cultures: the soluble CSF-1 receptor may play a role analogous to those that promote the stability of various circulating hormones. As such, binding of sCSF-1R to CSF-1 may increase the ligand's half-life and, therefore, its effects as a systemic regulator. The binding affinity of membrane-bound receptors may be greater than that of sCSF-1R and, therefore, allow for an efficient exchange of CSF-1 as the sCSF-1R/CSF-1 complex arrives at the target cell.

The presence of a control mechanism based on sCSF-1R secretion for the modulation of CSF-1 activity is novel for this colony-stimulating factor. Soluble forms for several cytokine receptors have already been identified, including those for IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, G-CSF, GM-CSF, type I and II IFNs, TNF, GH, Epo, and LIF (19-26); the mode of action of some of these was described in detail in Chapter I. These soluble receptors can result from alternative splicing of the mRNA species, which leads to the production of truncated proteins that have the extracellular ligand binding regions of the receptor, but lack the cellular membrane anchoring domains. Examples of this include IL-4Rα, IL-5Rα, IL-6Rα, IL-7Rα, IFNαR β chain, and the GM-CSFRα. Alternatively, the full-length membrane-associated receptor can be proteolytically cleaved and released from the cell surface as has been shown for IL-2Rα, GM-CSFRα, TNFRI, and TNFRII. To date, there are no examples whereby the soluble receptor was encoded by a distinct gene from the membrane-associated form of the receptor.

The soluble CSF-1R was encoded by a unique full-length transcript that encodes for the extracellular domain of mCSF-1R, but not for the transmembrane or cytoplasmic tyrosine kinase domains. The identification of this transcript suggests that PKM sCSF-1R was generated through alternative splicing of the c-*fms* gene, although the presence of a distinct gene encoding sCSF-1R cannot be discounted. Support for the alternative spicing hypothesis comes mainly from examination of sequence homologies between mCSF-1R and sCSF-1R; their transcript sequences were 100% homologous between the start codon and residues 619. Interestingly, the 3' end of this identical region corresponds to the predicted exon4-intron4 (E4-I4) boundary. Whereas the mSCF-1R open reading frame continues by splicing exon 5 at this point, the sCSF-1R appears to result from continued transcription through the E4-I4 boundary. Observation of the sequence in this region indicates that there were significant differences with the consensus recognition sequence for Exon-Intron splicing [AG | GT(A/G)AGT; where underlined residues were essential for the recognition process] (27). Deviations from this consensus sequence have been implicated with decreased efficiency of splicing. One could therefore hypothesize that the deviation from the splicing recognition consensus sequence at the E4-I4 boundary leads to continued transcription of the growing CSF-1R mRNA into intron 4. This coupled to the presence of polyadenylation signals closely downstream from the E4-I4 boundary may result in the generation of an alternatively spliced form of the CSF-1 receptor, a soluble form that only encodes for the extracellular ligand-binding domain of the membrane-bound full-length receptor.

It remains unclear whether this novel form of the CSF-1R and the negative feedback mechanism we have postulated above is conserved. However, examination of the intron-exon boundaries in the two species where the complete c-*fms* gene has been characterized, human (U63963) and puffer fish (U63926), was consistent with such evolutionary conservation. In fact, analysis of the intron-exon boundaries in these two species, for the regions corresponding to the ligand-binding domain encoded by the c-*fms* gene indicated that there are a number of marked divergences from the consensus sequence described above. Moreover, intronic polyadenylation signals are present downstream from some intron-exon boundaries, particularly in the human c-*fms* gene. As a result there is a strong possibility that future studies may yield additional alternatively-spliced transcripts of the CSF-1R gene, each with differential affinities for the CSF-1 ligand.

The anti-sCSF-1R antibody conclusively showed the presence of native sCSF-1R in PKM culture supernatants. The lowest concentration of sCSF-1R was detected in the *proliferative* phase, shown in Chapter V to be associated with proliferation and differentiation events at the peak of PKM growth. The progressive increase in sCSF-1R concentration between *early proliferative* and *senescence* phases of PKM development, together with the recombinant sCSF-1R data, supported my hypothesis that this molecule actively contributed to the decrease of proliferation events associated with entrance of PKM into the *senescence* phase. The presence of sCSF-1R in serum containing medium and *lag* phase supernatants suggested a possible systemic regulatory role for sCSF-1R in the goldfish. Interestingly, sCSF-1R concentration decreased significantly between *lag* and *early proliferative* phases' PKM supernatants suggesting the presence of macrophage-mediated endogenous control mechanisms for sCSF-1R activity.

The generation of the anti-sCSF-1R antibody will be essential to the subsequent characterization of the CSF-1/CSF-1R regulatory system. In addition, because sCSF-1R shares the ligand-binding domain with the mCSF-1R we are assessing whether this polyclonal antibody may also recognize mCSF-1R of the surface of developing PKM, and may serve as a marker of macrophage differentiation. Finally, the anti-sCSF-1R antibody and the recombinant sCSF-1R may be used in subtraction experiments aimed at the isolation and characterization of a CSF-1-like molecule from PKM cultures, thus fulfilling a critical role in the future characterization of macrophage hematopoiesis in fish.

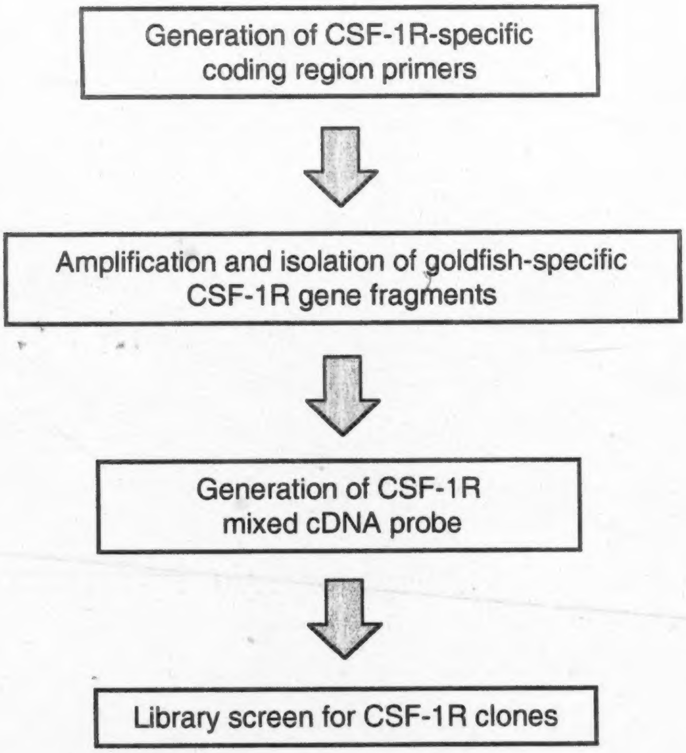

Generation of CSF-1R-specific coding region primers
Amplification and isolation of goldfish-specific CSF-1R gene fragments
Generation of CSF-1R mixed cDNA probe
Library screen for CSF-1R clones

Figure 6.1: Identification of the CSF-1R transcript in primary kidney macrophages.

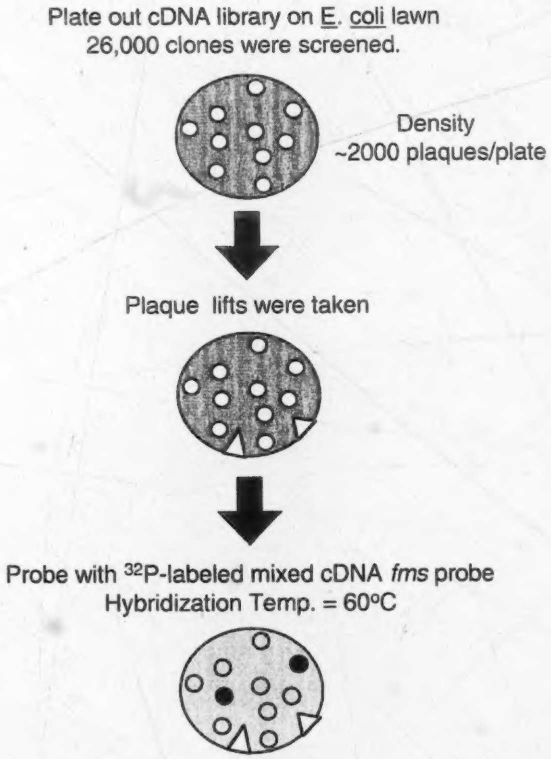


Plate out cDNA library on E. coli lawn
26,000 clones were screened.
Density
~2000 plaques/plate
Plaque lifts were taken
Probe with ^{32}P-labeled mixed cDNA *fms* probe
Hybridization Temp. = 60°C

Figure 6.2: Primary screen: identification of CSF-1R positive clones. Schematic representation shows the primary screen for CSF-1R (*fms*) clones. Transcripts from *proliferative* phase (14,000 clones) and *senescence* phase (12,000 clones) cDNA libraries were analyzed. Primary screen yielded eight putative *fms* clones. These were subsequently analyzed via a PCR-based secondary screen.

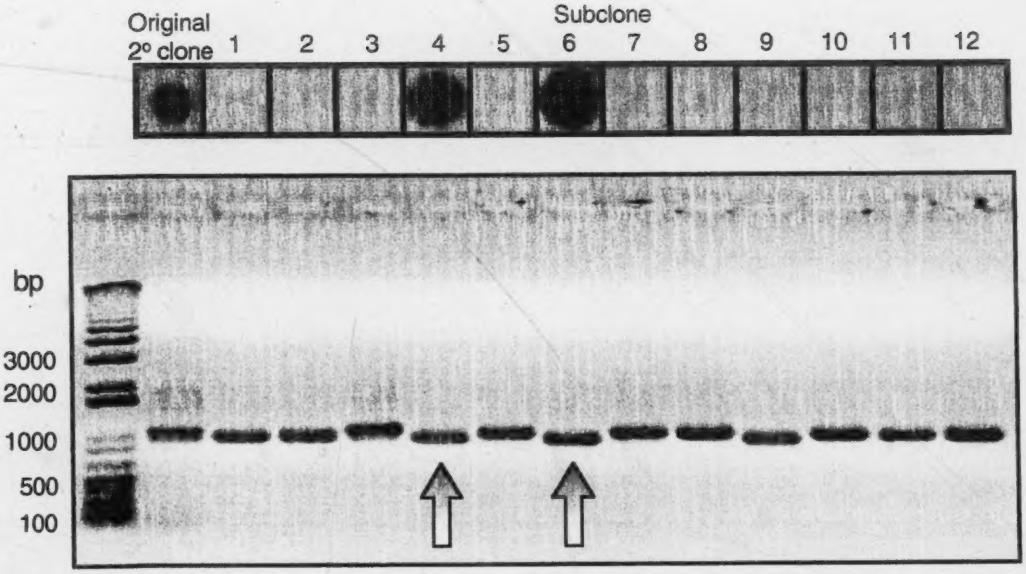

Original
2° clone
Subclone
1
2
3
4
5
6
7
8
9
10
11
12
bp
3000
2000
1000
500
100

Figure 6.3: Tertiary screen: identification of CSF-1R positive subclones. Following the secondary screen, CSF-1R positive clones were replated and individual subclones were isolated. These subclones were PCR-amplified and individually arrayed onto a gridded nylon membrane, which was subsequently screened with the *fms*-specific mixed cDNA probe. Results for a representative secondary screen isolate are shown. The tertiary screen confirmed the specific binding of the original secondary clone to the *fms*-probe. Note that two of the isolated subclones encoded for *fms*-positive single-sized transcripts.

```
D.rerio      MFFALLFLIGILL-GQVQGWSEPRIRLSSGALAGTDVILESGSPLQLVCEGDGPVTFLPR 59
C.auratus    M-FALLFVCGILF-AQAQGWSEPRIRLNSEDPVGTDVILDSGSPLHLICEGDGPVTWLPR 58
T.rubripes   MQSFLPLLMGIMASASSVEWRHPVIWFNSKVVQSSEVVVKPGTSLELKCGGDGPVNWQTR 60
H.sapiens    MGPGVLLLLLVAT-----AWHGQGIPVIEPSVP--ELVVKPGATVTLRCVGNGSVEWDGP 53
             *   : :: :          *     * . .        ::::..*:.: * * *:*.* :

D.rerio      LAKHKRYISKEVGKIRSFRVEKTTVDFTGTYKCVYMNGNDS-NLSSSVHVFVRDSRVLFV 118
C.auratus    LAKHKRYISKEVGNVRSFYVKQATADFTGTYKCVYMNTNDS-NESSSVHVFVRDNRFLFV 117
T.rubripes   LPKHKRYMSRSPGNLRTIRVARPTAEFTGTYKCFYSAWAQHRHLTSSVHVYVKDPNRVFW 120
H.sapiens    ASPHWTLYSD--GSSSILSTNNATFQNTGTYRCTEPG--DPLGGSAAIHLYVKDPARPWN 109
              . *    *    *.   : . ..* : ****:*        :     ::::*::*:*     :

D.rerio      SPSTSLRYVRKEGEDLLLPCLLTDPEATD-FTFRMDNGSAAPYGMNITYDPRKGVLIRNV 177
C.auratus    SPSTSLRYVRKEGEDLTLPCLLTDPDATD-FTFRMDNGSAVPYGMNVTFDPKKGVLIRNV 176
T.rubripes   TSSTSLRVVRKEGEDYLLPCLLTDPEATD-LGLRMDNGTTVPPEMNYTVYRHRGILIRSL 179
H.sapiens    --VLAQEVVVFEDQDALLPCLLTDPVLEAGVSLVRVRGRPLMRHTNYSFSPWHGFTIHRA 167
                : . *  *.:*  ********     . :   .*        * :    :*. *:

D.rerio      HPGFNADYICCARIGGAEKVSKIFSINIIQRLRFPPYVYLKRNEYVKLVGERLQISCTTN 237
C.auratus    HPGFNADYVCSARIRGVWKVSKIFSINVIQRLHFPPYVFLKRSEHVKLVGERFQISCTTN 236
T.rubripes   QPSFNADYVCTAKVKGVEKTSKTFSINVIQKLRFPPYVFLEMDEYVRIVGEELQIRCMTH 239
H.sapiens    KFIQSQDYQCSALMGGRKVMSISIRLKVQKVIPGPPALTLVPAELVRIRGEAAQIVCSAS 227
             :    . ** * * : *     * : ::: : :  ** : *    * *:: **  ** * :

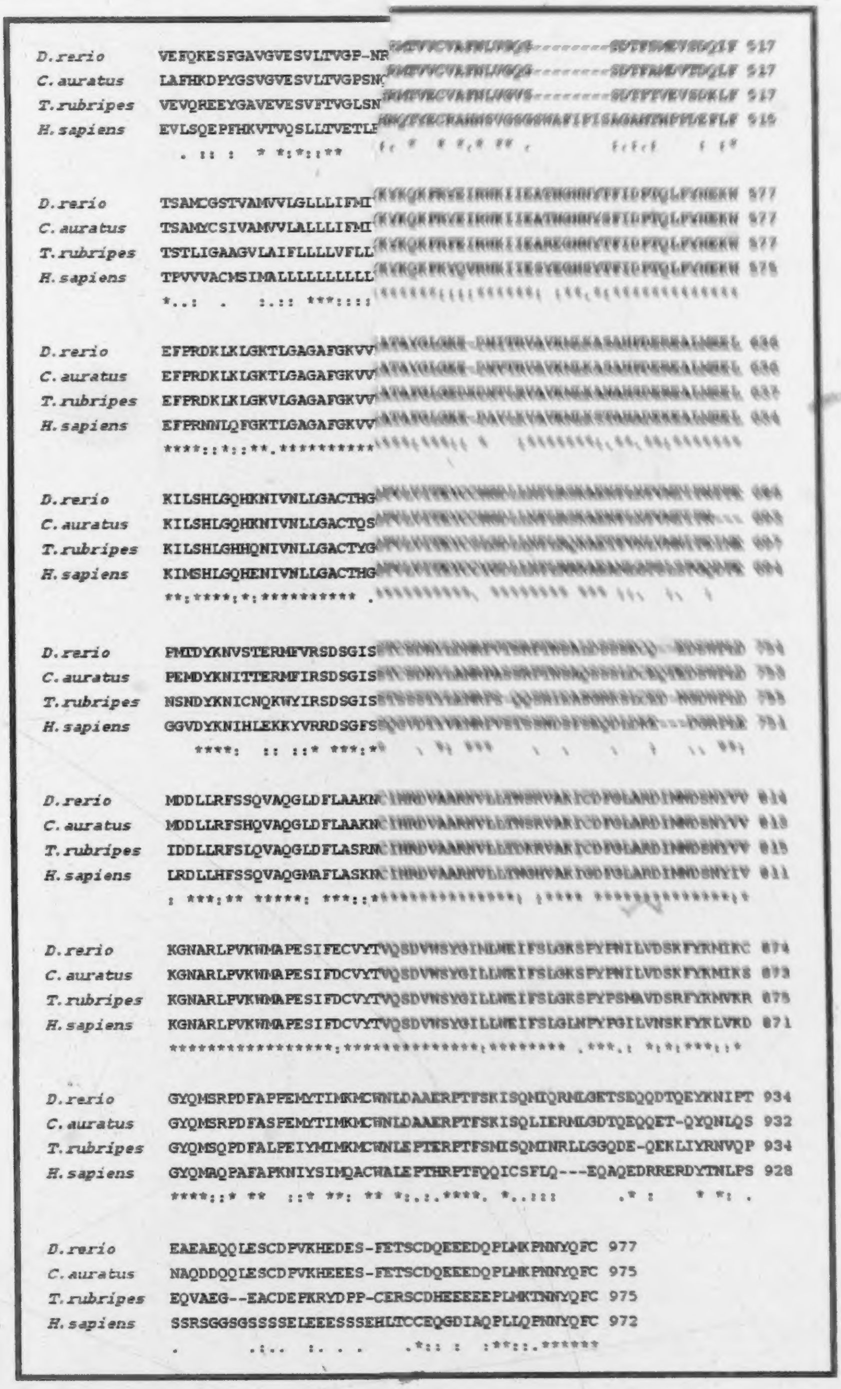
D.rerio      NPNFYYNVTWTHSSRMLPKAEEKSTMEGD-RLAIESILTIPSVQLSHTGNITCTGQNEAG 296
C.auratus    NPNFYYNVTWTHSSRTLPRAEEKSTMEGD-RLAIESILIIPAVQLSDSGNITCTGQNEAG 295
T.rubripes   NPNFNYNVTWNYTTKSRVTIEERVRSSGENRLDIQSILTISAVDLADTGNISCIGTNEAG 299
H.sapiens    SVDVNFDVFLQHNN-TKLAIPQQSDFHNN-RYQKVLTLNLDQVDFQHAGNYSCVASNVQG 285
             . :. ::*   :..        ::    .: *       * :  *:: .:** :* . *  *

D.rerio      ANSSTTQLLVVEEPYIRLSPKLSSKLTHRGLSIEVSEGDDVDLGVLIEAYPPLTSHKWET 356
C.auratus    ANSSTTQLLVMEEPYIRLSPKLSSKVTHRGLSIEVKEGEDVDLRVLIEAYPPLISHKWVT 355
T.rubripes   VNSSNTYLLVVEKPYIRLWPQLIPKLASQGLSVEVNEGEDLELGVMVEAYPQITDHRWHT 359
H.sapiens    KHSTSMFFRVVESAYLNLS-------SEQNLIQEVTVGEGLNLKVMVEAYPGLQGFNWTY 338
              :*:.  : *:*..*:.*          : :.*  **. *:.::* *::**** : ...*

D.rerio      PTSHNASLPENRFF----NHNDRYEALLLLKRLNFEEIGQYTLNVKNSMKSASITFDIKM 412
C.auratus    PTSHNASLPENRFY----NYNDRYEALMFLKRLNFQETGRYTLHVNSSMKNASIAFDIKM 411
T.rubripes   PTSPSTSMQEH-----------IYHARLQLKRMNAQEQGQYTFYAKSNLANGSISFHVKM 408
H.sapiens    LGPFSDHQPEPKLANATTKDTYRHTFTLSLPRLKPSEAGRYSFLARNPGGWRALTFELTL 398
               . .    *              :   : * *:: .* *:*:: ...      :::*.:.:

D.rerio      YTKPVARVKWENVT---TLSCRSYGYPAPSILWYQCTGIRTTCPENTTDLQP---IQTQT 466
C.auratus    YKKPVAMVRWENVT---TLSCRSYGYPAPSILWYQCTGIRTTCPENSTGLQP---VQTQT 465
T.rubripes   YQKPIAVVRWENIT---TLTCTSFGYPAPQIIWYQCSGIRPTCNGNNTGLPKQNHPQALT 465
H.sapiens    RYPPEVSVIWTFINGSGTLLCAASGYPQPNVTWLQCSGHTDRCDEAQVLQVWD---DFYP 455
                 * . * *  :.   ** * : *** *.: * **:*   *     .        :. .
```

Figure 6.4: Deduced amino acid sequence alignment of goldfish, zebrafish, puffer fish, and human CSF-1R. ClustalX multiple alignment of the goldfish CSF-1R predicted amino acid sequence with that of known CSF-1Rs determined the location of identical (*) and similar residues (. or :). Corresponding Genbank accession numbers for *D. rerio*, *T. rubripes*, and *H. sapiens* are AAF76872, AAC60063, CAA27300, respectively.



```
D.rerio      VEFQKESFGAVGVESVLTVGP-NR
C.auratus    LAFHKDPYGSVGVESVLTVGPSN
T.rubripes   VEVQREEYGAVEVESVFTVGLSN
H.sapiens    EVLSQEPFHKVTVQSLLTVETLF
               . :: :  * *:*::**

D.rerio      TSAMCGSTVAMVVLGLLLIFMI
C.auratus    TSAMYCSIVAMVVLALLLIFMI
T.rubripes   TSTLIGAAGVLAIFLLLLVFLL
H.sapiens    TPVVVACMSIMALLLLLLLLLL
             *..:  .   :.:: ***::::

D.rerio      EFPRDKLKLGKTLGAGAFGKVV
C.auratus    EFPRDKLKLGKTLGAGAFGKVV
T.rubripes   EFPRDKLKLGKVLGAGAFGKVV
H.sapiens    EFPRNNLQFGKTLGAGAFGKVV
             ****::*::**.**********

D.rerio      KILSHLGQHKNIVNLLGACTHG
C.auratus    KILSHLGQHKNIVNLLGACTQS
T.rubripes   KILSHLGHHQNIVNLLGACTYG
H.sapiens    KIMSHLGQHENIVNLLGACTHG
             **:****:*:********** .

D.rerio      PMIDYKNVSTERMFVRSDSGIS
C.auratus    PEMDYKNITTERMFIRSDSGIS
T.rubripes   NSNDYKNICNQKWYIRSDSGIS
H.sapiens    GGVDYKNIHLEKKYVRRDSGFS
                ****:  :: ::* ***:*

D.rerio      MDDLLRFSSQVAQGLDFLAAKN
C.auratus    MDDLLRFSHQVAQGLDFLAAKN
T.rubripes   IDDLLRFSLQVAQGLDFLASRN
H.sapiens    LRDLLHFSSQVAQGMAFLASKN
             : ***:** *****: ***::

D.rerio      KGNARLPVKWMAPESIFECVYTVQSDVWSYGILLWEIFSLGKSPYPNILVDSKFYKMIKC 874
C.auratus    KGNARLPVKWMAPESIFDCVYTVQSDVWSYGILLWEIFSLGKSPYPNILVDSKFYKMIKS 873
T.rubripes   KGNARLPVKWMAPESIFDCVYTVQSDVWSYGILLWEIFSLGKSPYPSMAVDSRFYKMVKR 875
H.sapiens    KGNARLPVKWMAPESIFDCVYTVQSDVWSYGILLWEIFSLGLNPYPGILVNSKFYKLVKD 871
             ******************:***************:********* .***.: *:*:***::*

D.rerio      GYQMSRPDFAPPEMYTIMKMCWNLDAAERPTFSKISQMIQRMLGETSEQQDTQEYKNIPT 934
C.auratus    GYQMSRPDFASPEMYTIMKMCWNLDAAERPTFSKISQLIERMLGDTQEQQET-QYQNLQS 932
T.rubripes   GYQMSQPDFALPEIYMIMKMCWNLEPTERPTFSMISQMINRLLGGQDE-QEKLIYRNVQP 934
H.sapiens    GYQMAQPAFAPKNIYSIMQACWALEPTHRPTFQQICSFLQ---EQAQEDRRERDYTNLPS 928
             ****::* **  ::* **: ** *:.:.****. *..:::      .* :    * *: .

D.rerio      EAEAEQQLESCDPVKHEDES-FETSCDQEEEDQPLMKPNNYQFC 977
C.auratus    NAQDDQQLESCDPVKHEEES-FETSCDQEEEDQPLMKPNNYQFC 975
T.rubripes   EQVAEG--EACDEPKRYDPP-CERSCDHEEEEEPLMKTNNYQFC 975
H.sapiens    SSRSGGSGSSSSELEEESSSEHLTCCEQGDIAQPLLQPNNYQFC 972
             .       .:..  :. . .     .*:: :  :**::.******
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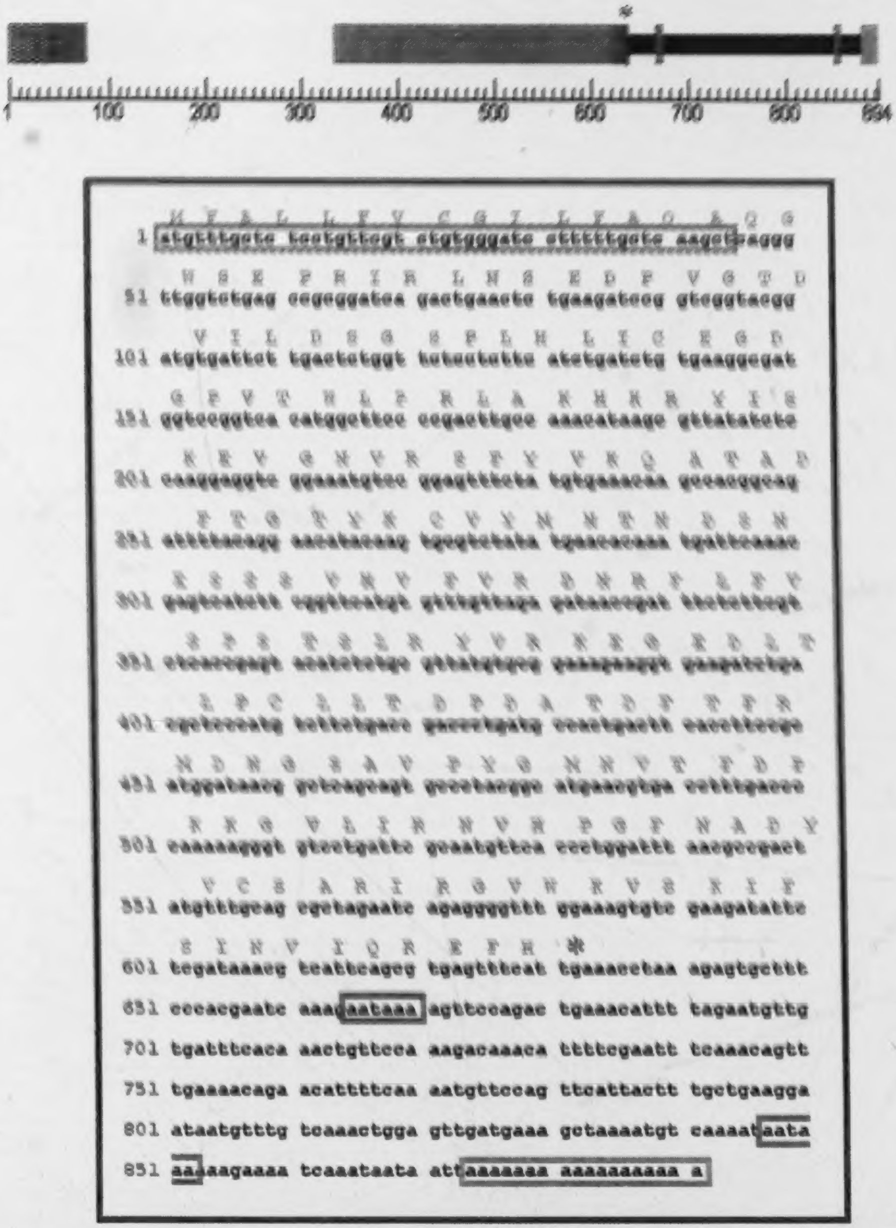

Figure 6.5: Identification of a putative soluble form of the CSF-1 receptor. Sequence analysis of the *fms*-positive primary kidney macrophage transcript determined that it only encoded for the extracellular portion of the CSF-1R. The isolated transcript represented a full-length mRNA species as it contained a start codon (ATG), signal peptide (dotted red box), stop codon (blue asterisk), polyadenylation signals (green boxes), and a poly-A tail (orange box). Based on homology with the human c-*fms* sequence, this novel transcript only encodes for exons 2, 3, and 4 (red, yellow, and blue boxes, respectively), and an additional unique 3' end (purple box).

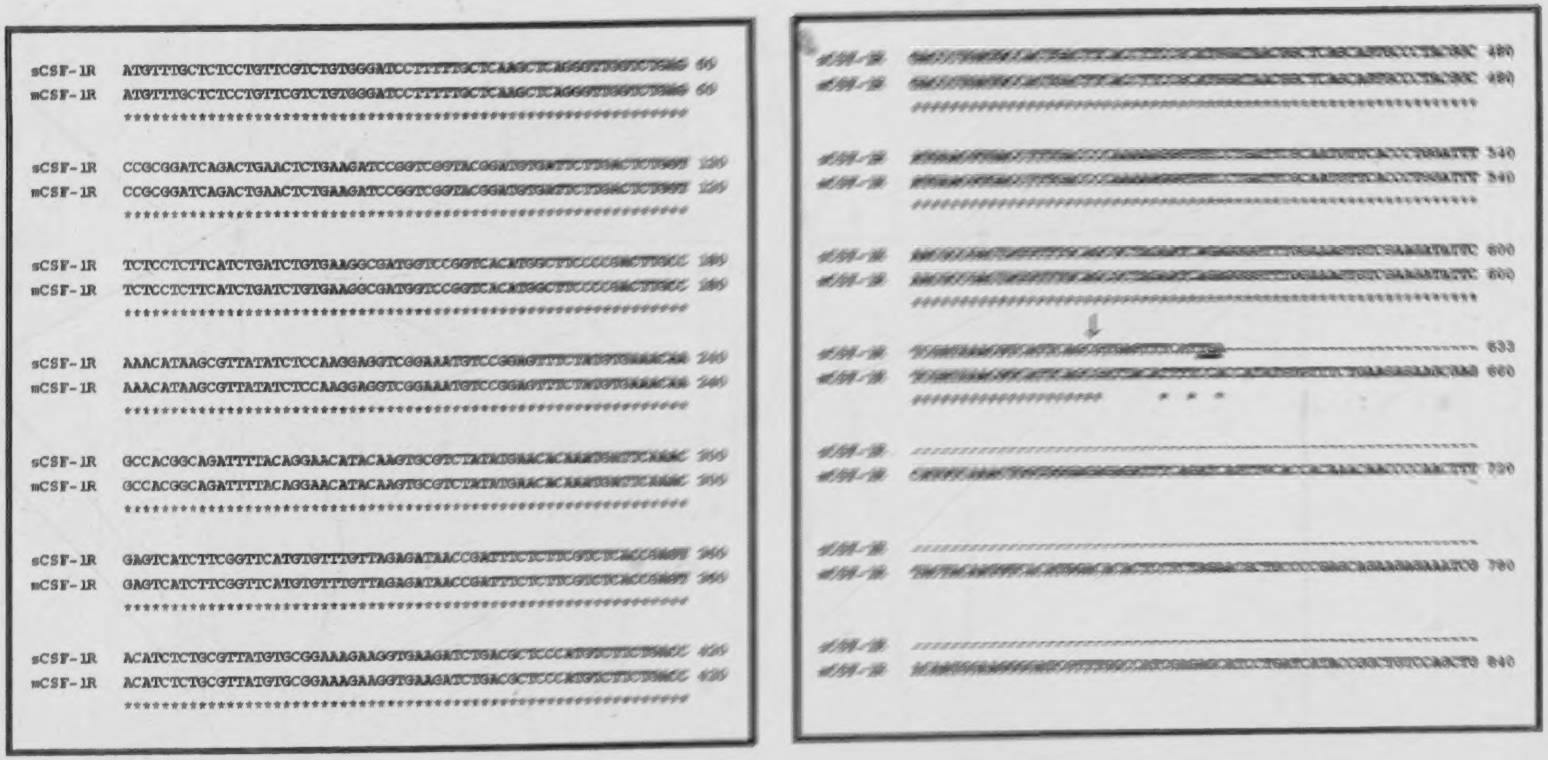
sCSF-1R
mCSF-1R
60
120
180
240
300
360
420
480
540
600
633
660
720
780
840

Figure 6.6: Alignment of goldfish membrane-bound CSF-1R (mCSF-1R) and the unique senescence phase short CSF-1R transcript. The nucleotide sequence spanning the open reading frame (ORF) of the short PKM CSF-1R transcript was aligned with the 5' region of the mCSF-1R ORF. Note that there is 100% homology between the ATG start codon and residue 619, which corresponds to the predicted exon 4-intron 4 boundary (↓).

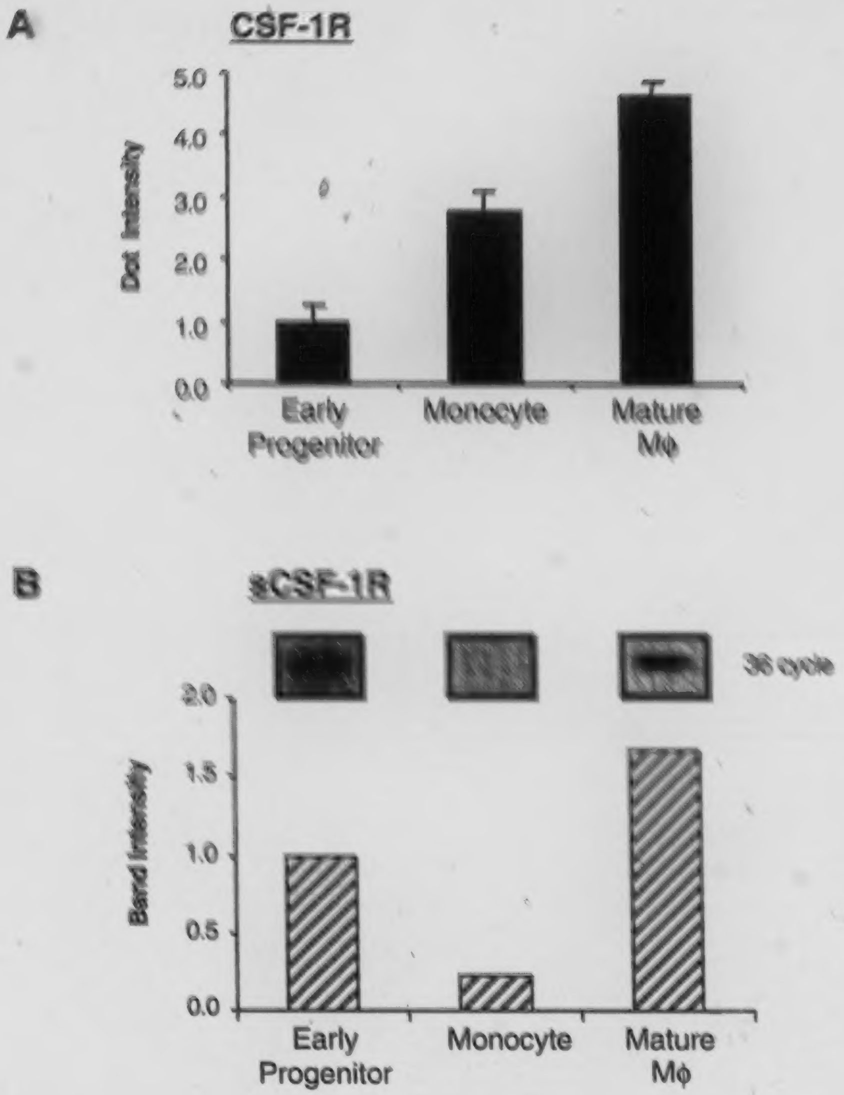

Figure 6.7: Characterization of CSF-1 receptor mRNA expression. A) Macroarray analysis indicated that CSF-1R transcripts could be readily detected in all three PKM subpopulations, with a progressive increase in gene expression corresponding to the more mature stages along the macrophage lineage. B) Semi-quantitative RT-PCR analysis, using primers directed against the soluble form of the PKM CSF-1R (sCSF-1R), indicated that this transcript was preferentially expressed in the two self-renewing PKM subpopulations, the R1-early progenitors and the R2-mature macrophages. Values are based on triplicate readings corresponding to each subset and were normalized to the early progenitor subpopulation (value=1) in order to determine the relative level of expression between the three stages of differentiation. Mϕ: mature macrophage.

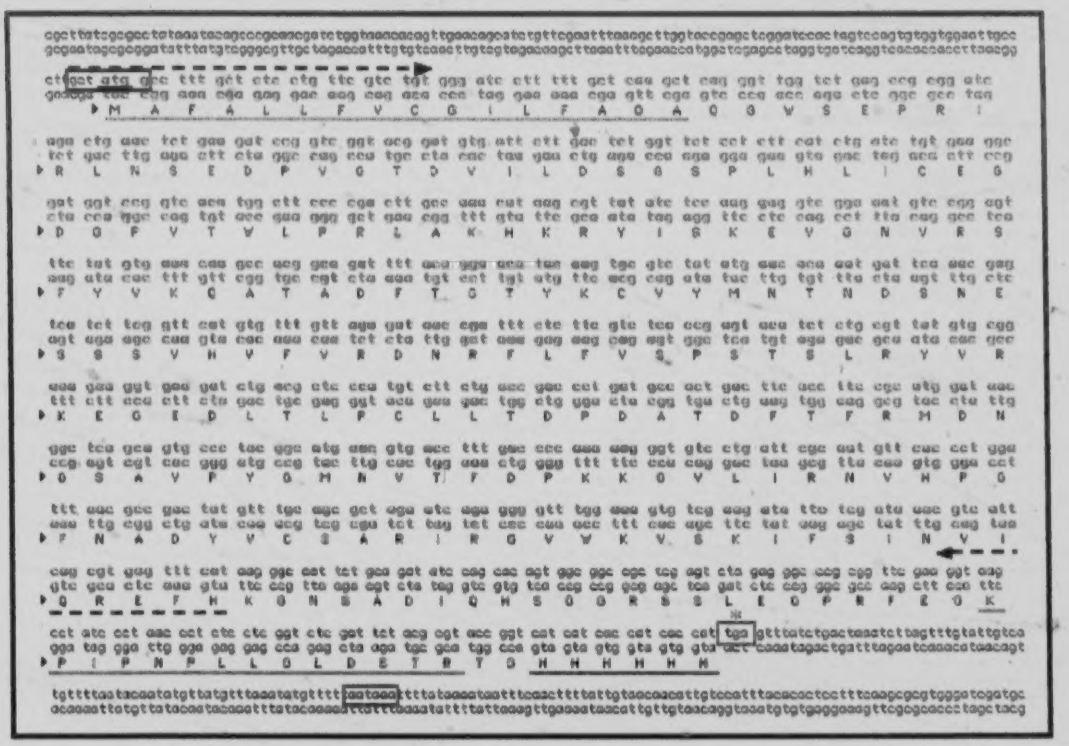

Figure 6.8: Design of the sCSF-1R protein expression construct. Schematic representation shows the sCSF-1R expression insert following cloning into the pIB/V5-His-TOPO vector. The sCSF-1R open reading frame (ORF) is shown in blue and the predicted amino acid sequence is presented in capitals below the nucleotide sequence. The pIB/V5-His-TOPO vector sequence is presented in red. The sCSF-1R expression insert included a Kozak sequence-flanked start codon (red box) and signal peptide (green underline). The native stop codon was removed in order to allow for translation of the 3' V5-epitope (orange underline) and 6xHis tag (purple underline), to be used in the detection and purification of the recombinant protein, respectively; a vector-derived stop codon (blue asterisk) was maintained in-frame downstream on sCSF-1R insert. Expression primers flanking the sCSF-1R ORF, used in PCR-amplification of the expression insert, are denoted by a dotted line.

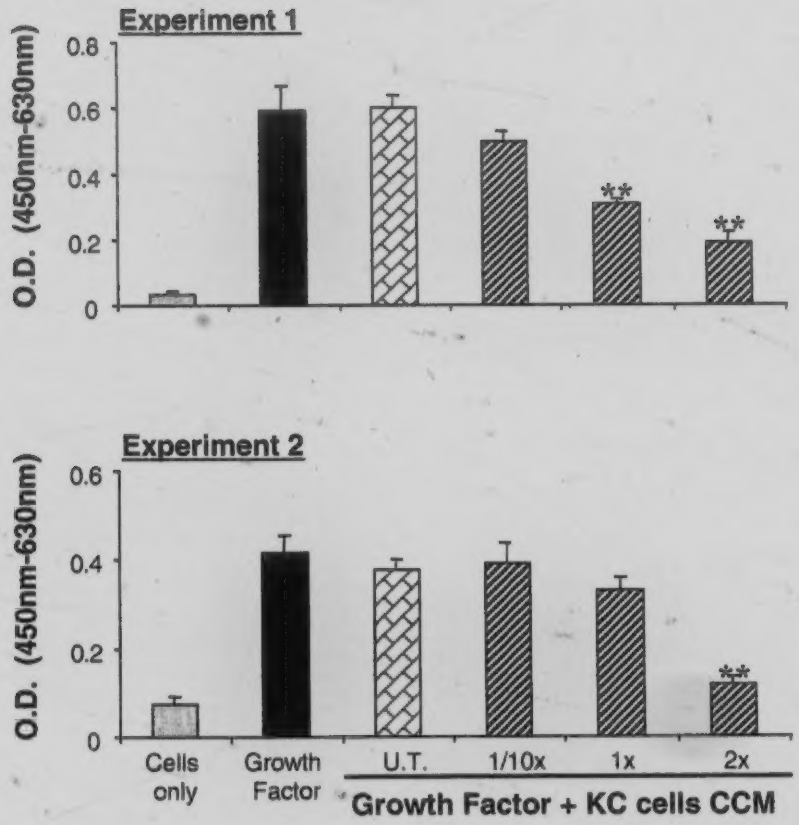

Figure 6.9: Inhibition of PKM proliferation by transformed KC cell supernatants. Kidney leukocytes were cultured in the absence (grey bars) or presence (black bars) of PKM supernatants containing high MGF activity. PKM growth in the presence of PKM supernatants (black bars) was compared to PKM growth in the presence of PKM supernatants treated with untransformed KC cell CCM (bricked bars), or increasing concentrations of supernatants from KC cells expressing recombinant sCSF-1R (hatched bars). Note the dose-dependent decrease in proliferation as a result of treatment with recombinant sCSF-1R. The amount of cell proliferation following each treatment was quantitated using the Cell Proliferation ELISA BrdU incorporation analysis. Each bar represents the mean ± SEM of triplicate cultures. The experiment was repeated on kidney leukocytes isolated from 3 fish; representative results from 2 fish are shown. Significant differences between cells grown in the absence (black bar) or presence (hatched bars) of sCSF-1R are marked by a double asterisk ($P < 0.01$).

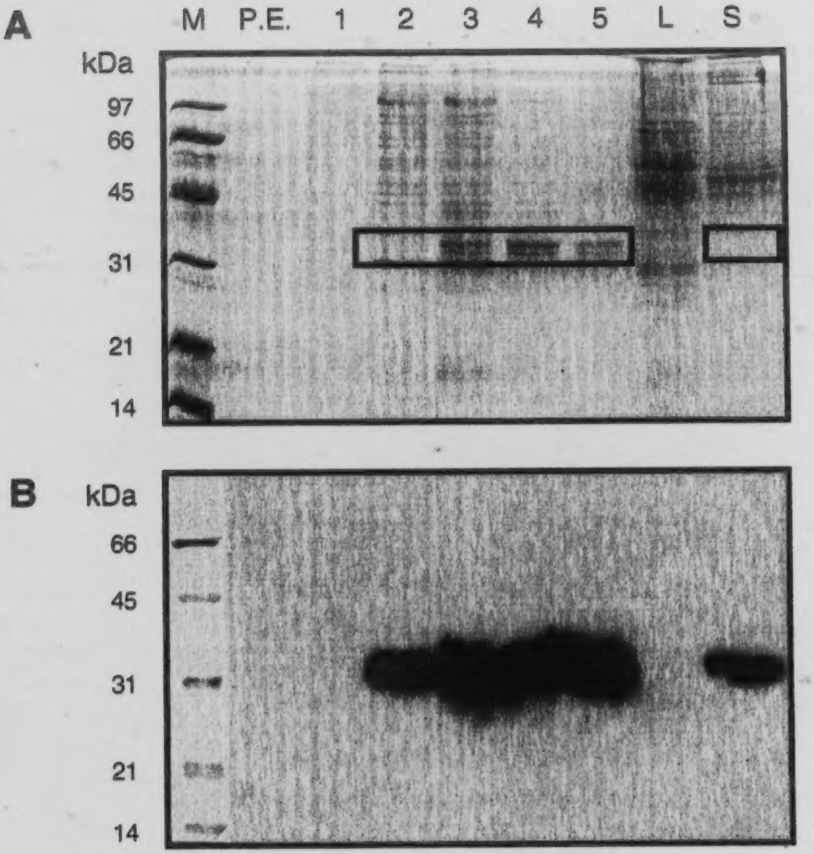

Figure 6.10: Purification of recombinant soluble CSF-1R (sCSF-1R). Blastacidin-selected KC cells were assessed for expression of recombinant sCSF-1R. Analysis of KC cell lysates and culture supernatants confirmed that the recombinant protein was secreted (B). Supernatants were collected, dialyzed, and concentrated in preparation for His-tagged protein purification. Recombinant sCSF-1R was purified using Ni-NTA agarose columns, eluted with 125mM imidazole, dialyzed extensively against 1x D-PBS, and filter-sterilized in preparation for immunodetection and analysis of biological activity. A) Shows a Coomassie blue-stained 12.5% SDS-PAGE gel of the pre-elution fraction (P.E.), recombinant sCSF-1R purified fractions (1-5), transformed KC cell lysates (L), and transformed KC cell supernatants (S). B) Shows the corresponding western blot where recombinant sCSF-1R was immunodetected by an anti-V5 epitope monoclonal antibody. Highlighted bands on the SDS-PAGE gel correspond to those recognized by immunodetection.
M: molecular weight standards.

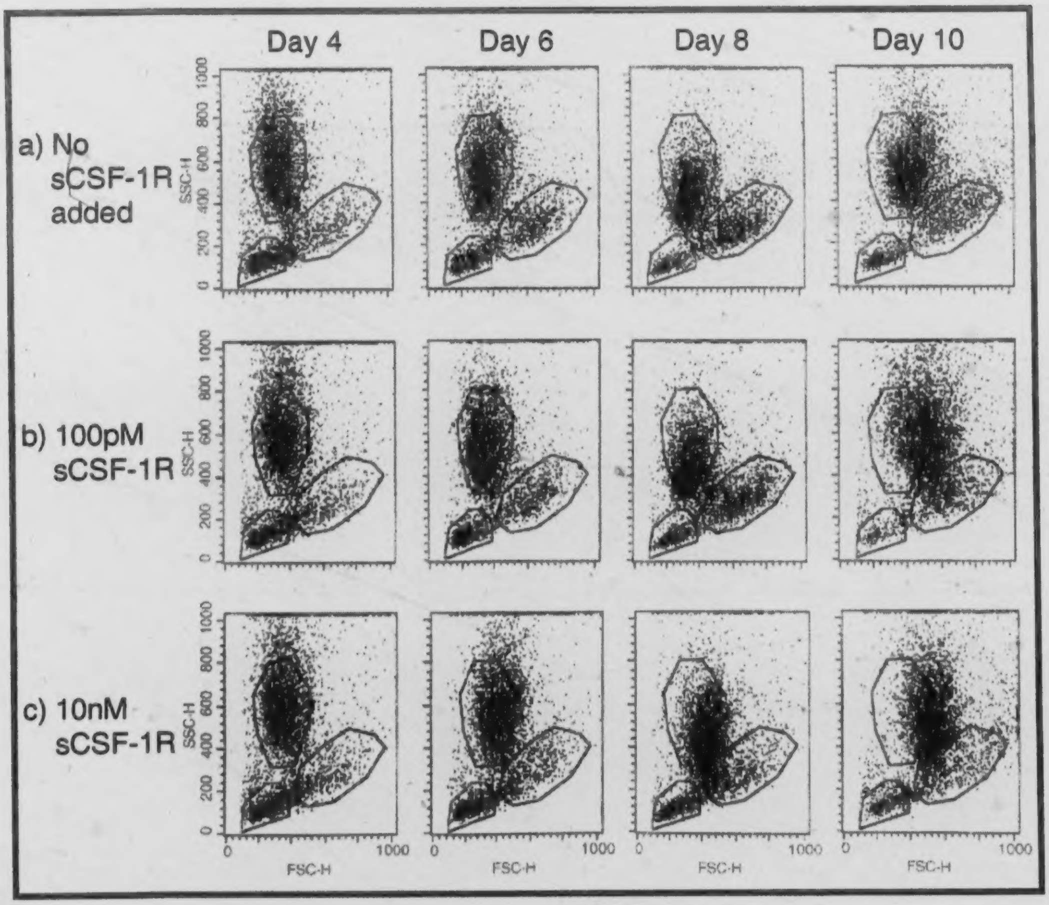

Figure 6.11: Effect of recombinant sCSF-1R on the growth-inducing activities of *proliferative* phase CCM. PKM cultures were grown for 4 days in the presence of *proliferative* phase CCM. Purified recombinant sCSF-1R was added in increasing concentrations to *proliferative* phase supernatants, and each mixture was used to treat one of the parallel PKM cultures at Day 4. The effect of each treatment on PKM proliferation was assessed during a 6-day observation period. Note the faster rate of PKM entrance into the *senescence* phase in those cultures treated with higher concentrations of recombinant sCSF-1R (compare to Fig. 5.6). Also note the less prominent mature macrophage subpopulation in PKM cultures treated with higher concentrations of recombinant sCSF-1R; for example, compare (a) and (c) on Day 8. The experiment was repeated on primary kidney macrophage cultures from 3 fish; representative results from one fish are shown.

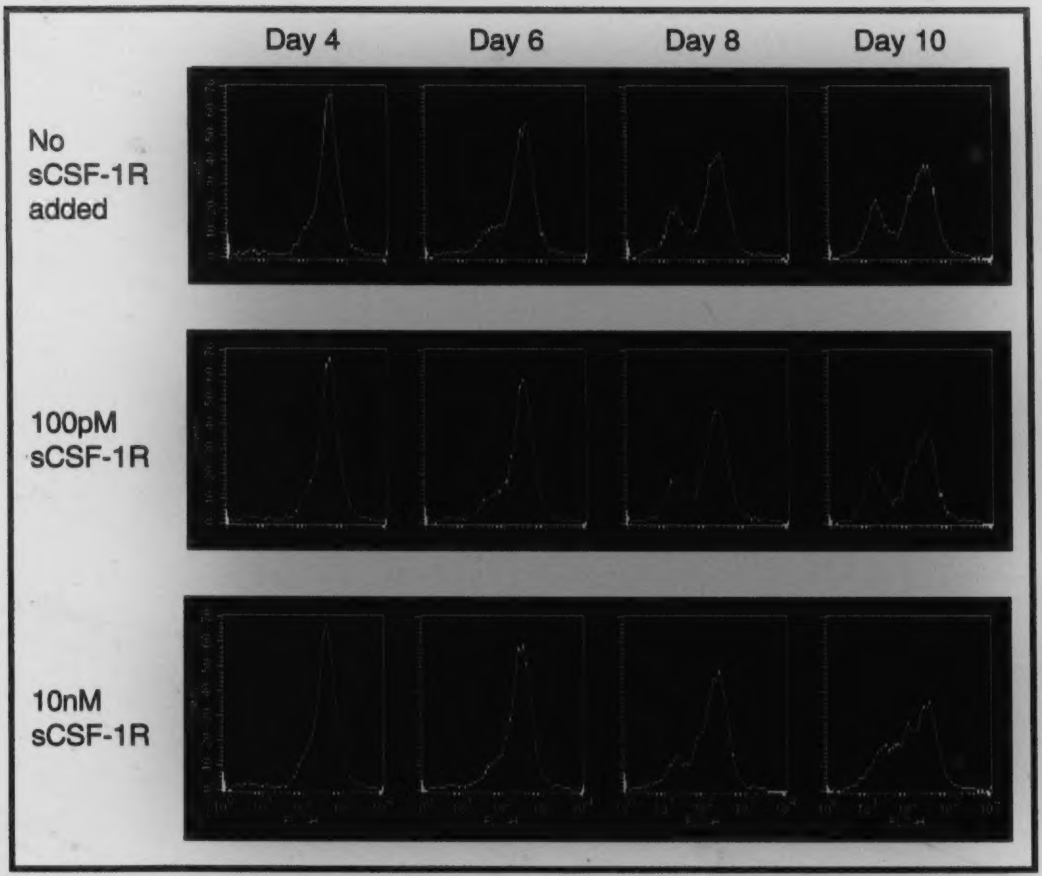

Figure 6.12: PKH26 analysis of PKM proliferation in response to recombinant sCSF-1R. PKM cultures were grown for 4 days in the presence of *proliferative* phase CCM. Purified recombinant sCSF-1R was added in increasing concentrations to *proliferative* phase supernatants, and each mixture was used to treat one of the parallel PKM cultures at Day 4. The effect of each treatment on PKM proliferation was assessed, using PKH26, during a 6-day observation period. Note the marked decrease in proliferation events in those cultures treated with higher concentrations of recombinant sCSF-1R (i.e. less prominent histogram shift to a lower fluorescence intensity). The experiment was repeated on primary kidney macrophage cultures from 3 fish; representative results from one fish are shown.

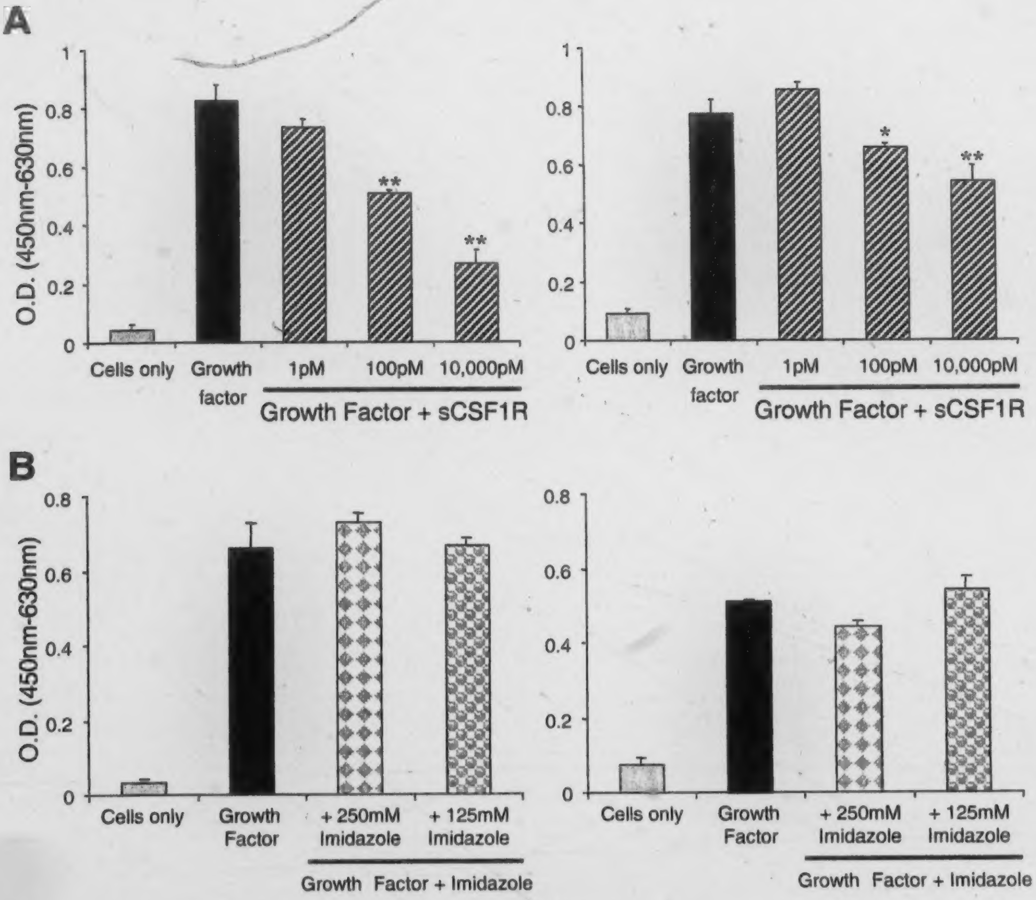

Figure 6.13: BrdU analysis of PKM proliferation in response to purified recombinant sCSF-1R. Cells were cultured in the absence (grey bars) or presence (black bars) of PKM supernatants containing high MGF activity. A) PKM growth in the presence of PKM supernatants (black bars) was compared to PKM growth in the presence of PKM supernatants treated with increasing concentrations of purified recombinant sCSF-1R (hatched bars). Note the dose-dependent decrease in proliferation as a result of treatment with recombinant sCSF-1R. Significant differences between cells grown in the absence (black bars) or presence (hatched bars) of sCSF-1R are marked by an asterisk ($P < 0.05$) or a double asterisk ($P < 0.01$). B) Imidazole, at concentrations above those used to elute recombinant sCSF-1R from Ni-NTA agarose columns was added to MGF-containing supernatants and dialyzed against 1x D-PBS just as the purified sCSF-1R shown in (A). The results show that imidazole was effectively removed through dialysis and did not affect PKM proliferation; no significant difference ($P > 0.05$) was observed between imidazole-treated supernatants and untreated controls. The amount of cell proliferation following each treatment was quantitated using the Cell Proliferation ELISA BrdU incorporation analysis. Each bar represents the mean ± SEM of duplicate cultures. Each experiment was repeated on kidney leukocytes isolated from 3 fish; representative results from 2 fish are shown.

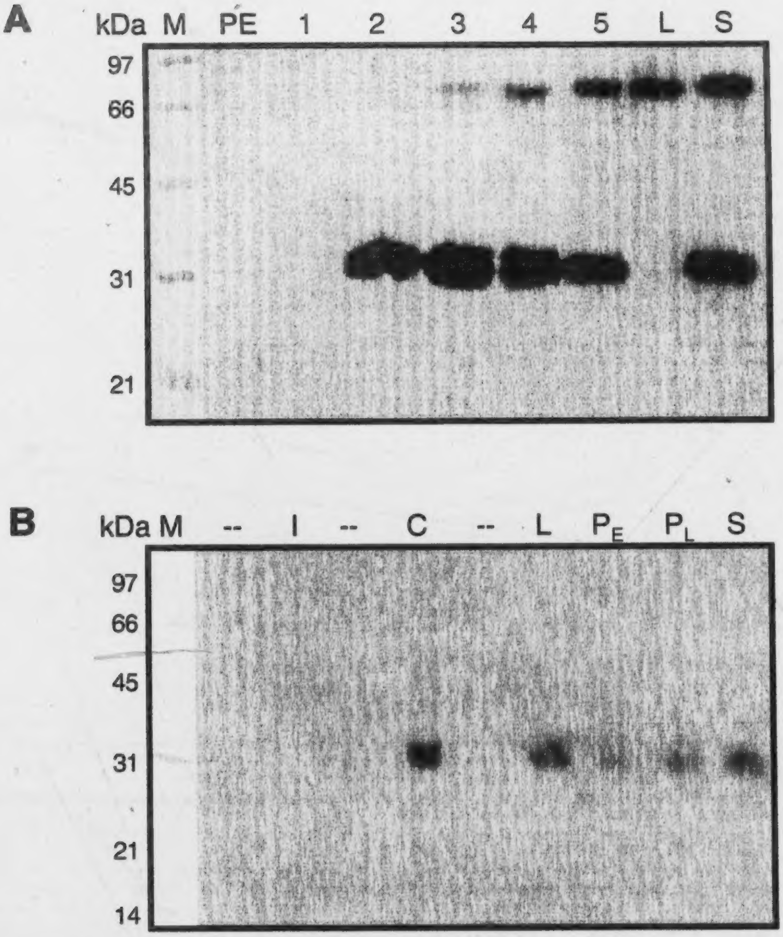

Figure 6.14: Generation of an anti sCSF-1R polyclonal antibody.
A) Blastacidin-selected KC cell lysates, supernatants, and Ni-NTA agarose column fractions were assessed for the presence of recombinant sCSF-1R as described in Fig. 6.10. In this case, however, the primary antibody utilized in the western blot procedure was a polyclonal antibody generated against purified sCSF-1R (fractions 4-5). Immunodetection of the ~31kDa band corresponds to the recombinant sCSF-1R previously detected using the anti-V5 epitope monoclonal antibody. In addition, the polyclonal antibody recognized an ~75kDa protein band. M: molecular weight standards, PE: pre-elution fraction, 1-5: recombinant sCSF-1R purified fractions, L: KC cell lysates, and S: KC cell supernatants. B) Analysis of culture supernatants from PKM in *lag* (L), *early proliferative* (P_E), *late proliferative* (P_L), and *senescence* phases (S) showed the kinetics of native sCSF-1R secretion. M: molecular weight standards, I: incomplete media (without serum), C: complete media (with serum), --:blank lane

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CHAPTER VII: IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES AS POTENTIAL MARKERS OF FISH MACROPHAGE DEVELOPMENT[6]

1. INTRODUCTION

The regulation of blood cell formation, or hematopoiesis, is a complex process whereby pluripotent stem cells are systematically guided to develop along one of the eight blood cell lineages (1-3). For cells of the macrophage lineage this process is generally associated with regulatory signals like the colony stimulating factors (CSFs) or other cytokines that either arrive from circulation or are produced locally by the surrounding hematopoietic microenvironment. Association of these regulatory signals with their cognate receptors on the surface of the developing cell activates the receptor and leads to the recruitment of an array of adaptor proteins, enzymes, and other intracellular signaling molecules that trigger specific downstream cascades (3, 4). Ultimately, all signal transduction pathways converge at the level of gene expression where positive and negative modulators of transcription interact and delineate the patterns of genes expressed by the cell and its overall hematopoietic response (5). It is these changes in gene expression that culminate in the modulation of protein production, the subsequent up-regulation of proliferation and differentiation events associated with stimulation of early progenitor cells with cytokines like IL-3 or GM-CSF, the induction of cellular commitment derived from discrete stochastic intracellular events, and ultimately the activation of negative feedback loops aimed at modulating the length and strength of the response to the initial stimulus. Such intricate control forms the basis for an orderly progression of cellular events and aberrations in these mechanisms are the underlying cause for diseases such as leukemias and lymphomas (6).

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[6] A version of this chapter has been submitted for publication:
Barreda, Hanington, Walsh, Wong, and Belosevic (2003) *Dev. Comp. Immunol.*
D.R. Barreda was involved in the data collection, analysis, and writing of the manuscript.

In this chapter, I described the identification of genes that may play an important role in the regulation of fish macrophage hematopoiesis, through the use of a differential cross-screen approach. Previous attempts to identify fish macrophage hematopoietic modulators using recombinant human and mouse cytokines proved unsuccessful, presumably due to limited cross-reactivity stemming from long evolutionary distances between species; similarly, our attempts to isolate cytokines, cytokine receptors, and a variety of macrophage differentiation markers by homology cloning techniques have had limited success (Chapter VI). Recently, we have seen the rapid identification of several fish genes that encode molecules of immunological interest, yet their immediate application to the understanding of fish immunity has been limited. This was because the high throughput gene sequencing/cloning procedures they were derived from provided little information about the cells that expressed them and the immune mechanisms that they contribute to.

The differential cross-screen approach allowed me to take advantage of the improved understanding of fish macrophage hematopoiesis gained from the detailed observation of primary kidney macrophage (PKM) behavior *in vitro*. I hypothesized that the shift from an actively proliferating and differentiating culture to one that enters a *senescence* phase and undergoes apoptotic cell death was a reflection of specific changes in gene expression. As a result, comparison of gene expression patterns between *proliferative* and *senescence* phases should lead to the identification of macrophage genes directly involved in the positive or negative regulation of hematopoietic events, as well as genes that are modulated down-stream from these regulatory points. To test these hypotheses, I differentially cross-screened a PKM cDNA library with probes from both *proliferative* and *senescence* phases. Using this approach, I have identified several candidate genes involved in fish macrophage hematopoietic regulation. I have also identified several potential markers of macrophage differentiation and function, and have characterized their expression patterns across three distinct stages of macrophage development. Additional transcripts were identified that may participate in signal transduction, ion regulation, protein processing, immune defense mechanisms, as well as several that encode for structural components and housekeeping enzymes. Therefore, I believe that future characterization of the putative hematopoietic regulators identified in this study will provide a more global understanding of macrophage biology, since they were examined in the context of expression analyses for a broad spectrum of genes.

2. RESULTS

2.1. cDNA library and screening

Figure 5.2 (Chapter V) showed the phenotypic changes associated with the shift of PKM cultures from the *proliferative* to *senescence* phases of PKM development. I have previously determined that during the *proliferative* phase most of the proliferation events and differentiation shifts occur, resulting in increased cell numbers and the appearance of three distinct stages of macrophage differentiation: early progenitors, monocytes, and "mature" macrophages (Chapters III, IV, V). In addition, analysis of the cell-conditioned media (CCM) growth-inducing capabilities indicated that maximum activity was consistently found in supernatants isolated from *proliferative* phase PKM cultures, thus suggesting that endogenous macrophage growth factor(s) (MGF) were predominantly produced during this phase (Chapter V). In contrast, as PKM cultures entered the *senescence* phase there was a progressive decrease in cellular proliferation, as well as an increase in cellular clumping, appearance of multinucleated giant cells, debris formation, cellular vacuolization, and cellular death though a process of apoptosis. Moreover, *senescence* phase CCM inhibited PKM growth suggesting the production of proliferation inhibitory signals during this phase of macrophage development (Chapter V). I hypothesized that the phenotypic and CCM activity profile changes that occur as cells shift from *proliferative* to *senescence* phases were a reflection of changes in the patterns of gene expression of PKM.

A *proliferative* phase cDNA library was constructed and used in the screening procedure (Fig. 7.1). We chose this cDNA library as it likely contained transcripts encoding for several hematopoiesis-related proteins that we were particularly interested in, such as those that induced the spontaneous growth of fish macrophages (MGF) as well as various macrophage differentiation markers. A cDNA library of 10^6 clones has >99% probability of including a copy of all rare transcripts (<10 copies per cell) at a detectable frequency (7). Thus, the cDNA library, which contained $>2\mathrm{x}10^6$ independent cDNA clones, was likely to contain representatives from high, medium, and low abundance transcripts involved in macrophage hematopoiesis.

Differential cross-screening of the proliferative phase cDNA library with *proliferative* and *senescence* phase ^{32}P-labeled cDNA probes identified several differentially expressed genes. Specifically, a primary screen of 9200 clones yielded 734 putative primary clones that were isolated and analyzed using a PCR-based secondary screen (Fig. 7.2). The majority of these clone isolates encoded a single transcript as determined by PCR amplification of the differential primary clones (data not shown). The secondary screen confirmed the differential expression of 306 clones (3.32% of the total number of screened clones).

2.2. Sequence analysis of differentially expressed transcripts

Sequence analysis of 158 transcripts preferentially expressed during the *proliferative* phase and 86 transcripts preferentially expressed during the *senescence* phase allowed their comparison to sequences previously deposited in the GenBank database. BLASTX analysis established these differential clones as orthologues to 136 known genes (Fig. 7.3A). The remaining 31 unique sequences displayed no significant similarity (e-value $>10^{-10}$) with sequences previously deposited in the GenBank database, thus defining a new group of potential regulators of macrophage development and/or function. Twenty-seven of the identified orthologues were found to encode for proteins with a putative role in hematopoiesis (Table 7.1B and Table 7.2), whereas the rest encoded for proteins with potential roles in various aspects of macrophage biology (Fig. 7.3B). Interestingly, several of the identified transcripts were homologous to genes previously characterized as markers of macrophage differentiation.

The majority of the characterized clones were singletons, as can be observed from the examples in Tables 7.1A and 7.2. Although it was expected that redundancy would increase as the number of differentially cross-screened and sequenced clones increased, the high proportion of singletons indicated that the constructed cDNA library provided proper coverage of PKM expressed genes and that the differential cross-screen technique was able to capture the complexity of changing gene expression profiles in developing macrophages. Sequence analysis also confirmed the reproducibility of the differential cross-screen results. In such cases where several clones encoded the same transcript, remarkable consistency was observed between the differential levels of gene expression of each clone as determined by the PCR-based secondary screen (Fig. 7.4), thus supporting its application as a viable technique for the accurate identification of fish macrophage differentially expressed genes.

2.3. Northern Blot analysis

Northern blot analysis provided an independent confirmation of the patterns of gene expression for several differential transcripts identified through the differential cross-screen technique. Comparisons between the gene expression profiles of three representative clones using both techniques were shown in Figure 7.5. Overall, we observed good correlation between the results obtained via the differential cross-screen technique and northern blot analysis, thus confirming the preferential expression of differential clones in either *proliferative* or *senescence* phases of fish macrophage development.

2.4. Identification of potential markers of macrophage differentiation and/or function

The progression of a developing hematopoietic progenitor cell along the macrophage lineage is dependent on the activation and silencing of several genes. Many of these reflect the decreased lineage plasticity exhibited by maturing macrophages, the increased receptivity of these cells to macrophage-specific factors, as well as the responses to overall changes in the immunological demands and the environment where developing macrophages reside. Our primary kidney macrophage cultures consisted of three distinct stages of macrophage differentiation that co-existed in a dynamic equilibrium under *in vitro* conditions. Each subpopulation displayed unique flow cytometric, morphological, and cytochemical characteristics, as well as unique functions (Chapter III). It was therefore not surprising that analysis of the differentially expressed clones determined that several orthologues had been previously described in the literature as markers of macrophage differentiation and/or function (Table 7.1B and Table 7.2).

Transcripts with the greatest potential to be used as markers in our immunological studies were selected for further analysis.

2.4.1 Macroarray analysis

Macroarray analysis allowed the examination of the gene expression profiles for all candidate markers of macrophage differentiation. Using this approach, the relative levels of gene expression of candidate transcripts were compared among the three stages of macrophage development; the results for the top ten candidates (Table 7.2) are shown in Figure 7.6 and a discussion of their gene expression profiles is presented below.

2.4.2 RT-PCR analysis

RT-PCR results closely correlated with those from macroarray analysis (Fig. 7.7) and those previously reported for known mammalian hematopoietic marker (Fig. 7.8). This supported the potential application of several genes identified in this study as markers of fish macrophage differentiation. In addition, RT-PCR allowed us to discriminate between various transcripts and their parent mRNA species (Fig. 7.7), providing a more accurate representation of their gene expression profiles. This was particularly useful in cases where several transcripts of related proteins were derived from the same pre-mRNA species.

Macroarray analysis and RT-PCR examined the differential expression of several genes across the three identified stages of PKM differentiation, thus confirming their potential use as markers of fish macrophage differentiation. For example, legumain, an asparaginyl endopeptidase central to class II MHC presentation of microbial antigens, increased significantly in the mature macrophage stage as previously described (8, 9). Transcription factor MafB, the molecular chaperone BiP/GRP78, as well as the widely described CD68 antigen (macrosialin), all known to progressively increase in response to macrophage maturation, also showed a significant increase in expression levels from early progenitors to the mature macrophage stage (10-13). Finally, our analyses further suggested the potential use of a number of transcripts as novel markers of macrophage differentiation (cathepsin H, aquaporin, Y-box protein 1, ATF4, BIP/GPR78; Fig. 7.6). These markers will complement other macrophage markers, including the central myeloid hematopoiesis regulator PU.1/ets-1, and the early hematopoietic progenitor markers c-kit and AC133 that have recently been identified in our laboratory through a variety of molecular techniques.

CD63 expression increased from PKM early progenitors to the monocyte stage as has been described in mammals; interestingly, it decreased significantly at the "mature" macrophage stage in contrast to previous reports that showed high levels of expression particularly in activated macrophages (14-16). Transcripts such as those encoding for IFN-inducible protein and nephrosin displayed a similar pattern of gene expression to CD63, and transcripts like that encoding for 14-3-3 protein displayed a contrasting pattern of gene expression (Fig. 7.6). We are currently examining whether these are a result of subset specific expression or are related to the alternate pathways of fish macrophage development we identified in PKM cultures (Chapter IV).

3. DISCUSSION

My approach employed the differential cross-screen technique (Fig. 7.1 and 7.2) and has successfully identified several potential hematopoiesis-related genes. A brief review of each of these differential genes and their role in the modulation of hematopoietic events, based on the literature, was presented on Table 7.1B. In each case, their functions were discussed as they may relate to the regulation of fish macrophage development. As predicted, a number of the genes preferentially expressed during the *proliferative* phase have been described to be involved in the induction, up-regulation, and/or progression of fish macrophage proliferation and/or differentiation events. In contrast, a number of the genes preferentially expressed during the *senescence* phase have been implicated in the inhibition and/or down-regulation of macrophage hematopoietic events. Many of the genes identified fulfill roles in cell cycle progression and overall cell growth, differentiation, signal transduction of growth factor stimuli, as well as in the inhibition or activation of apoptosis. In each case, their preferential expression in either *proliferative* or *senescence* phases of PKM development were consistent with previous reports identifying their functions as hematopoietic regulators (Table 7.1B). Our results clearly point to the inherent complexity of the hematopoietic regulatory machinery of the goldfish, and to the commitment that cells place in modulating the delicate balance of signals associated with the life-death equilibrium.

Some of the genes identified present attractive candidates for future studies. Granulin, the most prominent differentially expressed gene in this study, is a member of a highly conserved family of hematopoietic regulators that appear to pre-date the radiation of animals from plants (17). We have, therefore, initiated protein expression studies to more accurately examine the role of granulins in PKM development. Other transcripts appear to be members of common regulatory pathways and therefore warrant further examination; this is particularly true of cell cycle regulators such as the Zinc finger protein 147 and 14-3-3 protein (Table 7.1B). Moreover, transcripts like the one encoding for Rho-GDI β, may provide interesting insights into phenotypic changes observed in developing PKM cultures, such as the appearance of multi-nucleated giant cells.

The ribosomal protein family represents an interesting group of molecules identified in this study. A few examples are discussed on Table 7.1B. In recent years, several reports have described extraribosomal functions for many of these proteins that may have resulted from gene recruitment or gene sharing events (18-20). The changes in their specific patterns of gene expression in relation to other members of the translational machinery in the absence of increased ribosome biogenesis, as well as the specific changes of subcellular localization in response to a variety of stimuli point to secondary functions independent of protein biosynthesis. An increasing number of ribosomal proteins and translation initiation/elongation factors have been individually implicated in a variety of cancers, DNA damage repair, cell cycle regulation, cell differentiation, oogenesis, development, and apoptosis (18-25).

The robustness of the differential cross-screen approach was illustrated by the identification of a number of low abundance transcripts, in some cases not easily detected by subsequent northern blot and macroarray analyses. These included, for example, transcripts encoding for survivin, gRICH70, and block of proliferation-1 (Table 7.1A). However, the limited number of these clones identified, as well as the absence of cytokine or cytokine-like genes from our isolated differential transcripts illustrates one limitation of this approach: the differential cross-screen technique favored the identification of those genes that were differentially expressed between the *proliferative* and *senescence* phases and were expressed in relative high levels in either phase. These included, for example, clones encoding for CD63, the transcription factor MafB, as well as the granulin transcript. Granulin was preferentially expressed at high levels during the *proliferative* phase. The high level of expression was clearly evident when we compare the number of RT-PCR cycles required to amplify granulin versus other potentially important genes like C/EBPβ from the same template cDNA population (Fig. 7.7B). This high level of granulin gene expression increased both the number of clones encoding it in the screened cDNA library as well as the number of cDNA probe species that were specific for granulin during the procedure, thus facilitating the subsequent detection of granulin as a differentially expressed gene.

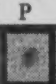
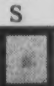
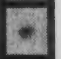
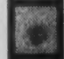
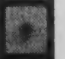
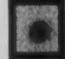
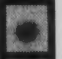
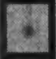
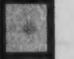
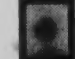
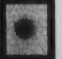
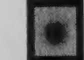
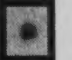
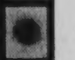
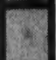
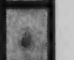
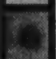
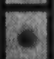
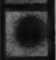
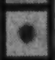
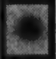
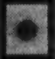
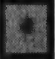
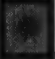
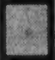
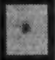
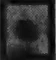
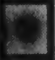
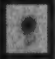
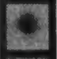
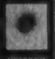
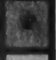
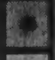
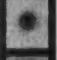
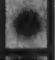
Cytokine genes, in contrast, were generally expressed in limited amounts, as femptomolar concentrations of active protein are generally sufficient to elicit strong responses. Moreover, expression of cytokine transcripts was expected to be under the stringent control of temporal regulatory mechanisms that include a variety of negative feedback loops as well as the presence of ARE-sequences that confer mRNA instability (4). As a result, cytokine transcripts were expected to be under-represented in the screened cDNA library and in the ^{32}P-labeled species used as mixed cDNA probes. However, it is important to note that despite the absence of cytokine genes we have identified several genes that are modulated by cytokines, such as legumain which is known to be specifically up-regulated in response to CSF-1- and not GM-CSF-induced macrophage differentiation (8). Thus, one may predict that a CSF-1-like molecule likely contributed to the development of our primary kidney macrophages.

It should be noted that the genes that we have identified represent but a small fraction of the genes involved in the regulation of fish macrophage hematopoiesis. The transcripts that we have isolated, however, represent important components of several unique pathways of hematopoietic control and therefore hint to mechanistic parallels between fish and mammals where such mechanisms may have already been thoroughly described. As such, this represents a critical first step in the generation of future strategies aimed at understanding macrophage hematopoiesis in fish as well as conservation of its regulatory mechanisms across evolution.

In addition to the putative modulators of hematopoietic events described above, we determined that several of the isolated clones encoded for previously described markers of macrophage differentiation and/or function. RT-PCR results closely correlated with those from macroarray analysis, supporting the potential application of several of these genes as markers of fish macrophage differentiation. In addition, RT-PCR allowed us to discriminate between various transcripts and their parent mRNA species, providing a more accurate representation of their gene expression patterns. Similar modifications in primer design have led to specific amplification of members within complex families of closely related mRNA species (data not shown). Therefore, we predict that subsequent analysis of specific candidates will take advantage of these features, particularly through Real-Time PCR applications, for the accurate examination of macrophage differentiation marker candidates.

The differential cross-screen approach used in this study has led to the identification of several putative modulators of macrophage development and well as several potential markers of macrophage differentiation. Currently, we are expressing several of these transcripts in both bacterial and insect protein expression systems, in order to further examine their role in the regulation of macrophage hematopoiesis as well as to generate antibodies that will be used in further characterizing the stages along the macrophage differentiation pathways. It is important to note that through this approach we have also identified several transcripts encoding proteins with putative roles at several levels of macrophage biology including signal transduction (e.g. STAT 3, SUMO-1), ion regulation (e.g. ATP-dependent Na+/K+ pump, voltage-dependent anion selective channel), protein processing (GTP-binding protein RAB2, Nedd ubiquitin protein ligases), immune defense mechanisms (hypoxia-inducible factor, ferritin), as well as various structural components and housekeeping enzymes. Therefore, we believe that future characterization of the putative hematopoietic regulators we have identified will provide a more global understanding of macrophage biology as they will be examined in the context of expression analysis for a broad spectrum of genes.

Table 7.1A: Identification of potential positive and negative regulators of fish macrophage hematopoiesis.

Clone	No. of Similar Clones	Preferential Expression [a] P	S	Putative Identity [b]	E-value [c]	Accession Number [d]
312	1			Regeneration-induced CNPase gRICH70	9e-62	U97682
243	1			Immunoglobulin heavy chain binding protein (BIP/GRP78)	1e-74	P06761
68	1			Zinc finger protein 147	1e-13	Q61510
97	1			14-3-3 protein	3e-75	JC5384
5	1			CHMP1	4e-52	AF281063
263	1			Nucleophosmin	5e-15	P07222
272	1			Adenine nucleotide translocator 2 (ANT2)	1e-29	AAB39266
147	31			Granulin	5e-58	AF273481
218.5	1			Survivin 1	7e-62	AY057057
456.5	1			STAT3	e-114	AJ005693
674	1			Ribosomal protein L18	9e-86	AF240374
237	1			Ribosomal protein P0	1e-91	NM_022402
54	1			Serine/Threonine kinase 3 (MST)	1e-64	BC010640
74	1			Block of Proliferation 1	3e-64	BC001086
472	1			Rho GDP-dissociation inhibitor 2 (Rho-GDI beta)	4e-40	P52566
550	1			Formin-like protein	5e-60	AF215666
589.1	1			APG5-like Apoptosis-specific protein	5e-31	NP_444299
547	1			Apoptosis Inhibitor 5 (FIF/AAC-11)	1e-92	XM_123850
522	1			Thymosin Beta 4	9e-15	AJ250180
567	1			Translation elongation factor 1 alpha	5e-94	S50143

[a] P=Proliferative phase, S=Senescence phase.

[b] Putative identities were based on sequence analysis of differential transcripts and subsequent comparison with GenBank database-deposited sequences using BLASTX analysis. Putative role in hematopoiesis regulation was based on literature descriptions of orthologue functions in various species.

[c] Listed clones showed an E-value below $1x10^{-10}$ in BLASTX search results. E-values were based on single-pass sequences ranging from 500-700bp.

[d] Accession number corresponds to the highest homology sequence based on BLASTX results.

Table 7.1B: Putative roles of identified differential genes in hematopoiesis regulation based on previously described orthologue functions.

Putative ID	Role in Hemopoiesis	Refs.
GRICH70	CNPase implicated in cellular regeneration processes.	(26, 27)
BIP/GRP78	Endoplasmic reticulum chaperone, related to heat shock protein family members, known to increase several-fold when M1 cells are induced to differentiate into macrophages by the treatment of IL-6. Moreover, it is known to exert protective functions against inflammation, reactive oxygen species, and apoptosis by blocking cytochrome c-mediated caspase activation.	(10, 28-30)
Zinc finger protein 147	Transcriptional regulator associated with induction of cell proliferation. Interestingly, this RING-finger-dependent ubiquitin ligase targets proteolysis of 14-3-3σ protein (see below), a negative cell cycle regulator known to cause G2 arrest, by enhancing proteasome-mediated turnover.	(31-33)
14-3-3 protein	Highly conserved family of proteins implicated as key regulators of signal transduction events. 14-3-3 has been shown to regulate entry into the cell cycle through interaction with Cdc25c, to interact with BAD preventing apoptosis by releasing Bcl-XL, and to regulate Raf-1, a key mediator of mitogenesis and differentiation. Moreover, 14-3-3 proteins have been reported to regulate the enzymatic activity of protein kinase C (PKC), a major family of serine/threonine kinases involved in cell growth and differentiation.	(34-40)
CHMP1	Member of structurally related family of proteins that plays a role in stable gene silencing within the nucleus and cell cycle progression.	(41)
Nucleophosmin	Major nucleolar phosphoprotein implicated as a positive signal for proliferation as well as an inducer of cellular differentiation. Moreover, it appears to play a significant role in mediating the anti-apoptotic effect of ras, a key component of apoptotic pathways.	(42-45)
ANT2	The adenine nucleotide translocator 2 gene is expressed in growth-activated cells and has been used as a marker of cell proliferation. Its expression is down-regulated in differentiating cells and those leaving the growth cycle. In addition, this ATP/ADP translocase also plays a significant role in the modulation of apoptosis initiation through interaction with Bcl-2 and Bax and the control of mitochondrial membrane permeability.	(46-52)
Granulin	Most readily isolated differentially expressed transcript in this study. Granulins are members of a unique and highly conserved family of cysteine-rich polypeptides with differential expression in hematopoietic cells. Granulins have been described to have an important role in modulation of cellular growth and differentiation. In fact, in several cell types the rate of proliferation is proportional to the level of granulin expression.	(17, 53-59)
Survivin 1	Bifunctional protein known to function at the interface between cell cycle progression and the control of apoptosis. Survivin 1 has been shown to play an important role in both oncogenic cell development and normal hematopoiesis. Up-regulation of expression occurs as a result of hematopoietic growth factor stimulation and is associated with increased progression through the cell cycle and inhibition of apoptosis. Conversely, growth factor withdrawal or survivin gene targeting results in inhibition of cell proliferation and increased apoptosis.	(60-68)
STAT3	Signal transducers and activators of transcription (STAT) proteins are a family of transcription factors with essential roles in the transmission of cytokine signals following receptor activation. STAT 3 is essential for gp130-mediated induction of proliferation, differentiation, and survival signals in response to stimulation with IL-6 or related cytokines.	(69-72)

Putative ID	Role in Hemopoiesis	Refs.
Ribosomal protein L18	L18 is a 60S ribosomal protein that has been associated with oncogenic progression in human colorectal cancer. It appears to mediate its actions through inhibition of dsRNA-activated protein kinase (PKR); PKR is known to provide a fundamental control step in the regulation of cell growth and differentiation, and the induction of apoptosis.	(18, 73)
Ribosomal protein P0	Identified as a DNA repair protein that may play a role in the regulation of apoptosis events through the control of DNA damage.	(18, 74)
Serine-Threonine kinase 3	This serine/threonine kinase is an orthologue of mammalian STE20-like kinase (MST), a physiological caspase substrate shown to play a positive role in the induction of apoptosis as an upstream activator and a downstream effector of caspase activity. Specifically, MST regulates death-receptor induced caspase activation and amplifies caspase-dependent morphological changes.	(75)
Block of Proliferation 1 (BOP1)	BOP1 is a conserved nucleolar protein involved in rRNA processing and ribosome assembly. Identified as an inhibitor of cellular proliferation from a mouse cDNA expression library, this protein appears to induce cell cycle arrest at G_1 and is under the control of p53.	(76, 77)
Rho-GDI β	Members of the Rho family of small guanosine triphosphatases have emerged as key modulators of actin cytoskeletal reorganization as well as cellular events such as cell cycle progression. Inhibition of Rho activity has been shown to induce cell cycle arrest at G_1. The Rho GDP-dissociation inhibitors (Rho-GDI) are central regulators of Rho activity by maintaining them in a GDP bound cytosolic inactive form. Furthermore, microinjection of Rho-GDI into *X. laevis* or sea urchin fertilized eggs inhibits cytokinesis without preventing nuclear division resulting in the formation of multinucleated eggs.	(78-80)
Formin-like protein	Formin-related proteins have been shown to play important roles in morphogenesis, cell polarity, and cytokinesis through interaction Rho family small GTPases. Moreover, expression of alternatively spliced transcripts leads to differential regulation of cell survival.	(81)
Apoptosis-specific protein (ASP)	Members of this family of proteins are dramatically up-regulated following induction of apoptosis. The marked increase in ASP protein levels are a result of regulation at the translational level as stimulation of apoptosis with various agents does not result in significant changes in mRNA levels. ASP does not appear to induce apoptosis; its expression is a relatively late event in the apoptotic process down-stream of caspase activation.	(82)
Apoptosis inhibitor-5	Apoptosis inhibitor 5 (FIF/AAC-11) is a ubiquitously expressed gene in embryonic and adult tissues. It encodes for a novel survival factor that inhibits apoptosis following growth factor withdrawal.	(83, 84)
Thymosin-β4	Thymosin-β4 has a ubiquitous localization and plays pleiotropic roles in both immune and non-immune cells. Its N-terminal domain encodes for AcSDKP, a known inhibitor of hematopoiesis. As such, thymosin-β4 has been shown to inhibit the growth bone marrow progenitors cells as well as that of granulocyte-macrophage and erythroid precursors following stimulation with a variety of growth factors.	(85-87)
EF-1α	Translational elongation factor 1α is a ubiquitously expressed member of the protein synthesis machinery that has been implicated in various aspects of hematopoietic regulation. Specifically, up-regulation of EF-1α expression leads to increased susceptibility to apoptosis-inducing signals in a role independent of protein synthesis. Conversely, hypo-expression of EF-1α correlates with increased resistance to apoptosis-inducing signals.	(18, 88, 89)

Table 7.2: Identification of potential markers of fish macrophage differentiation and/or function.

Clone	No. of similar clones	Putative identity [a]	E-value [b]	Accession Number [c]
471	1	Legumain	1e-64	AJ000990
24	1	CD63 antigen	5e-42	NP_031679
520	1	Macrophage antigen CD68 (Macrosialin)	8e-20	NM_001251
313	1	Cathepsin H	5e-52	BC002479
83	1	Interferon-inducible protein	9e-29	NM_001548
605	1	Aquaporin 7	4e-38	NM_007473
230	1	Y box protein 1 (YB-1)	5e-22	AB003335
553	1	Transcription factor ATF-4	9e-25	BAA76466
616	1	Transcription factor Maf-B	8e-12	AB006322
359	9	Nephrosin	7e-94	U62621

[a] Putative identities were based on sequence analysis of differential transcripts and subsequent comparison with GenBank database-deposited sequences using BLASTX analysis. Putative application as markers of macrophage differentiation was based on literature descriptions of orthologue expression profiles across maturing macrophage populations in various species.

[b] Listed clones showed an E-value below $1x10^{-10}$ in BLASTX search results. E-values were based on single-pass sequences ranging from 500-700bp.

[c] Accession number corresponds to the highest homology sequence based on BLASTX results.

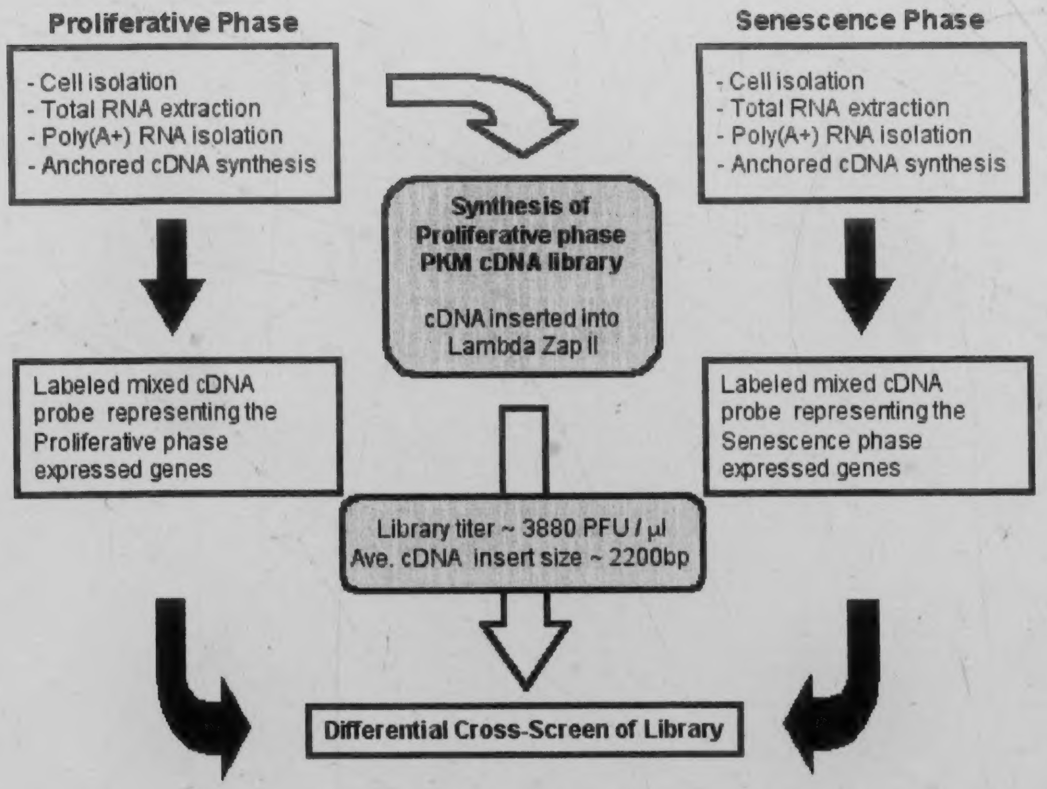
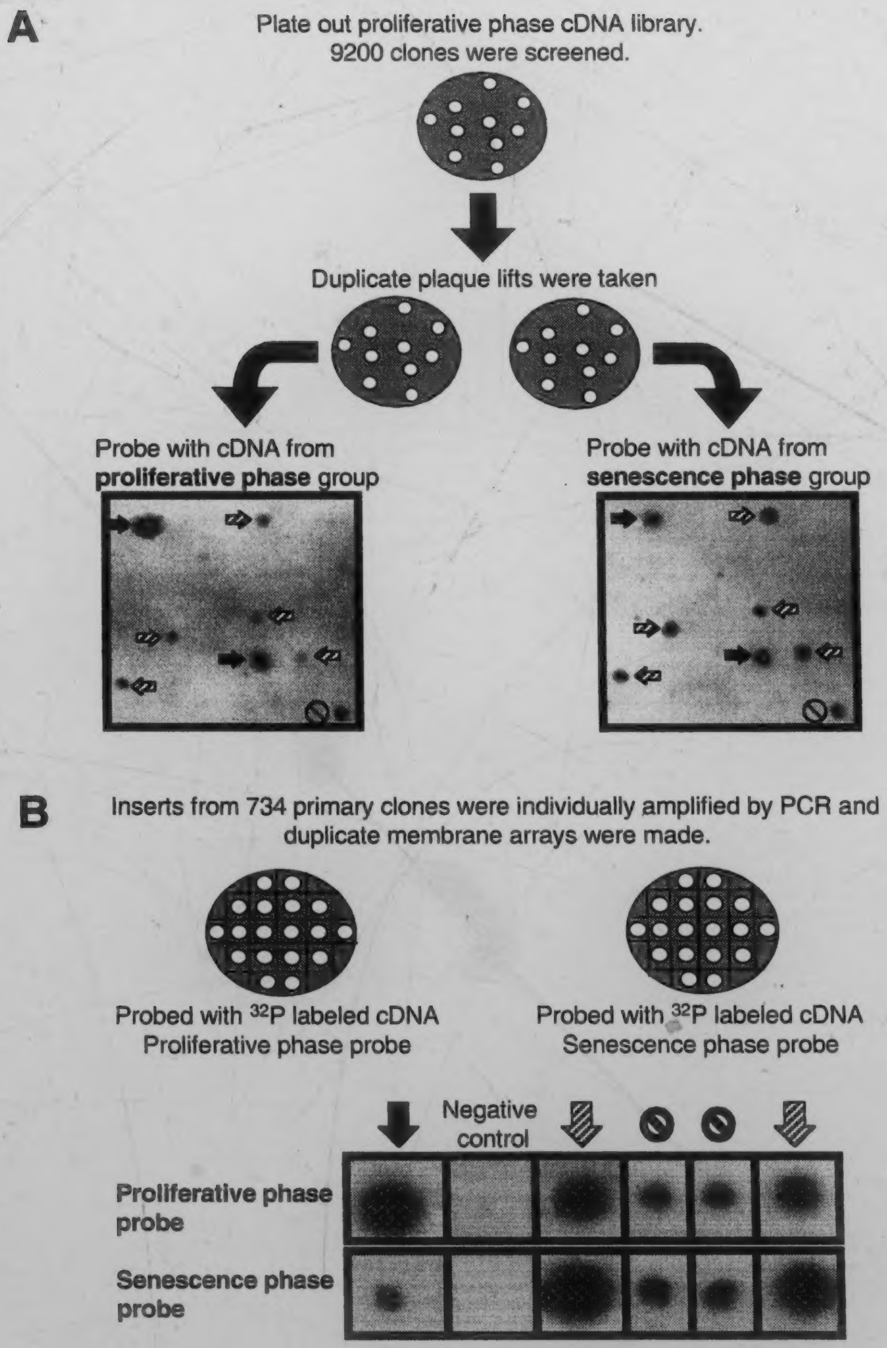

Proliferative Phase
- Cell isolation
- Total RNA extraction
- Poly(A+) RNA isolation
- Anchored cDNA synthesis
Labeled mixed cDNA probe representing the Proliferative phase expressed genes
Synthesis of Proliferative phase PKM cDNA library
cDNA inserted into Lambda Zap II
Library titer ~ 3880 PFU / µl
Ave. cDNA insert size ~ 2200bp
Senescence Phase
- Cell isolation
- Total RNA extraction
- Poly(A+) RNA isolation
- Anchored cDNA synthesis
Labeled mixed cDNA probe representing the Senescence phase expressed genes
Differential Cross-Screen of Library

Figure 7.1: Identification of genes preferentially expressed in the *Proliferative* and *Senescence* phases of macrophage development.

Figure 7.2: Differential cross-screen of proliferative phase cDNA library. Schematic representation shows A) the primary differential cross-screen of 9200 phage clones. A total of 734 putative differentially expressed primary clones were isolated. B) The secondary screen of all differentially expressed primary clones using a PCR-based dot screen confirmed the differential expression of 306 clones. Representative autoradiographs are shown. Black arrows depict those transcripts preferentially expressed during the proliferative phase of macrophage development, while stripped arrows depict those preferentially expressed during the senescence phase. The crossed circles depict those transcripts whose level of expression appears unchanged/consistent between both phases.


A
Plate out proliferative phase cDNA library.
9200 clones were screened.
Duplicate plaque lifts were taken
Probe with cDNA from
proliferative phase group
Probe with cDNA from
senescence phase group
B
Inserts from 734 primary clones were individually amplified by PCR and
duplicate membrane arrays were made.
Probed with ^{32}P labeled cDNA
Proliferative phase probe
Probed with ^{32}P labeled cDNA
Senescence phase probe
Negative
control
Proliferative phase
probe
Senescence phase
probe

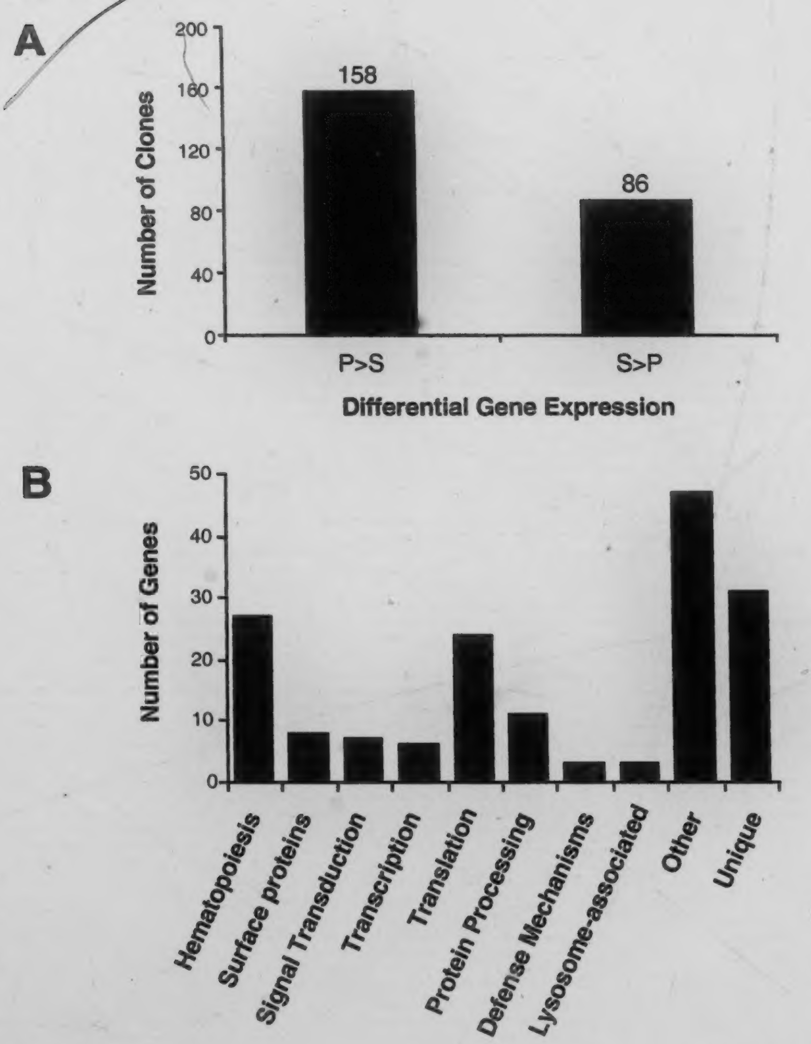


Figure 7.3: Summary of differential cross-screen results. Two-hundred forty-four clones, confirmed to be differentially expressed during the PCR-based secondary screen, were characterized using sequence analysis. A) The majority of differentially expressed transcripts were preferentially expressed during the proliferative phase of PKM development. B) Characterization of cloned transcripts determined that they encoded sequences with significant similarity (e-value < 10^{-10}) to 136 known genes, as well as 31 unique sequences that displayed no significant similarity with sequences previously deposited in the GenBank database. Sequences homologous to known genes were grouped according to their putative roles based on the literature. P: Proliferative phase, S: Senescence phase.

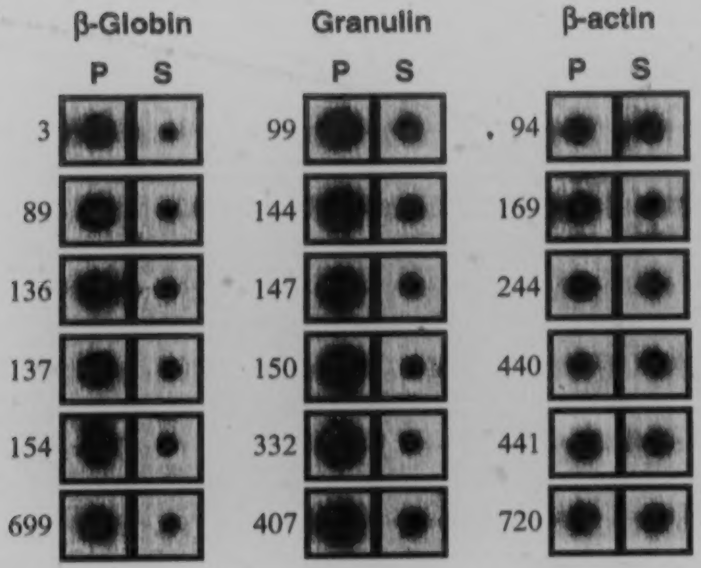

β-Globin
P
S
3
89
136
137
154
699
Granulin
P
S
99
144
147
150
332
407
β-actin
P
S
94
169
244
440
441
720

Figure 7.4: Reproducibility of differential cross-screen results following PCR-based secondary screen. Remarkable consistency was observed in the differential levels of gene expression between clones encoding the same transcript. Two representative differentially expressed transcripts encoded by several clones are shown (β-globin and granulin). The identity of the transcripts was based on subsequent sequence analysis. In addition, clones encoding beta-actin were isolated as a negative control, and displayed no significant difference in expression between the two phases of PKM development, thus supporting its utility as a control gene in our primary kidney macrophage system. P: Proliferative phase, S: Senescence phase.

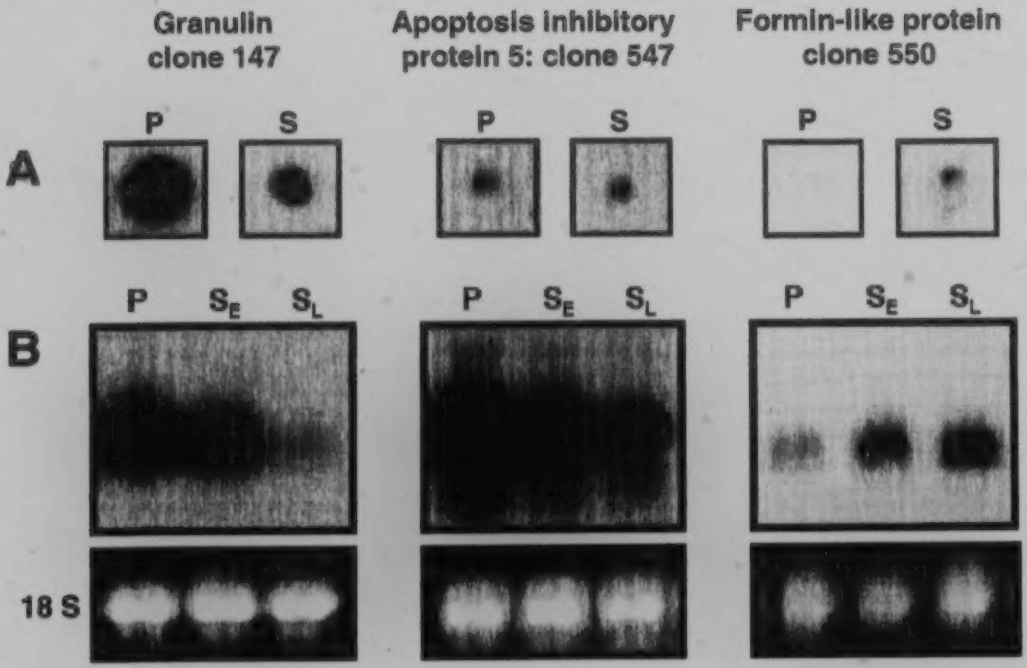

Granulin
clone 147
Apoptosis inhibitory
protein 5: clone 547
Formin-like protein
clone 550
A
P
S
P
S
P
S
B
P
S_E
S_L
P
S_E
S_L
P
S_E
S_L
18 S

Figure 7.5: Correlation between differential cross-screen and northern blots during analyses of PKM phases' gene expression profiles. Three representative clones were chosen and tested to assess the reproducibility of results based on two independent techniques. As can be observed, the trends in gene expression profiles are consistent between differential cross-screen (A) and northern blot (B) analyses. During the northern blot analysis the senescence phase was subdivided into early (S_E) and late (S_L) stages to further characterize the kinetics of gene expression. P: Proliferative phase, S: Senescence phase.

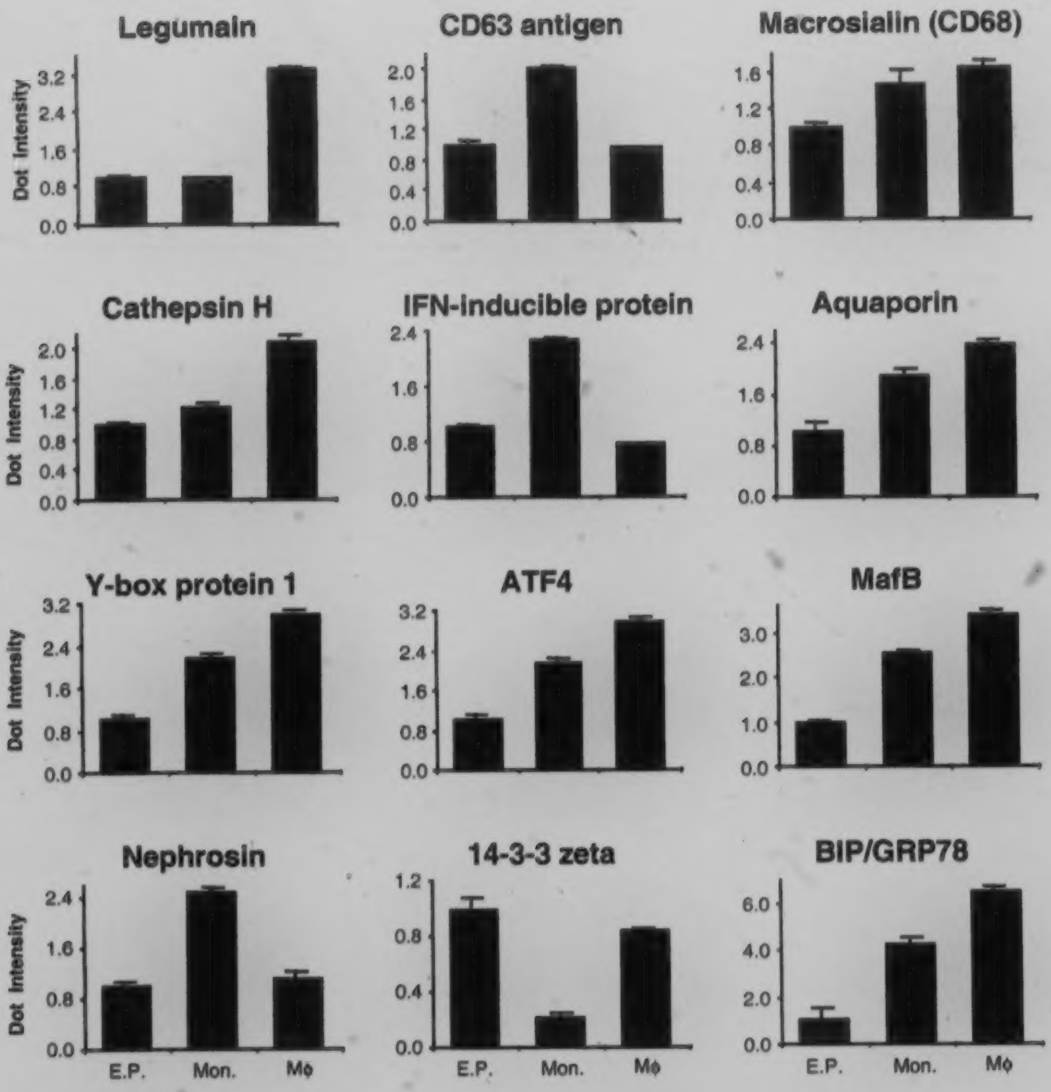
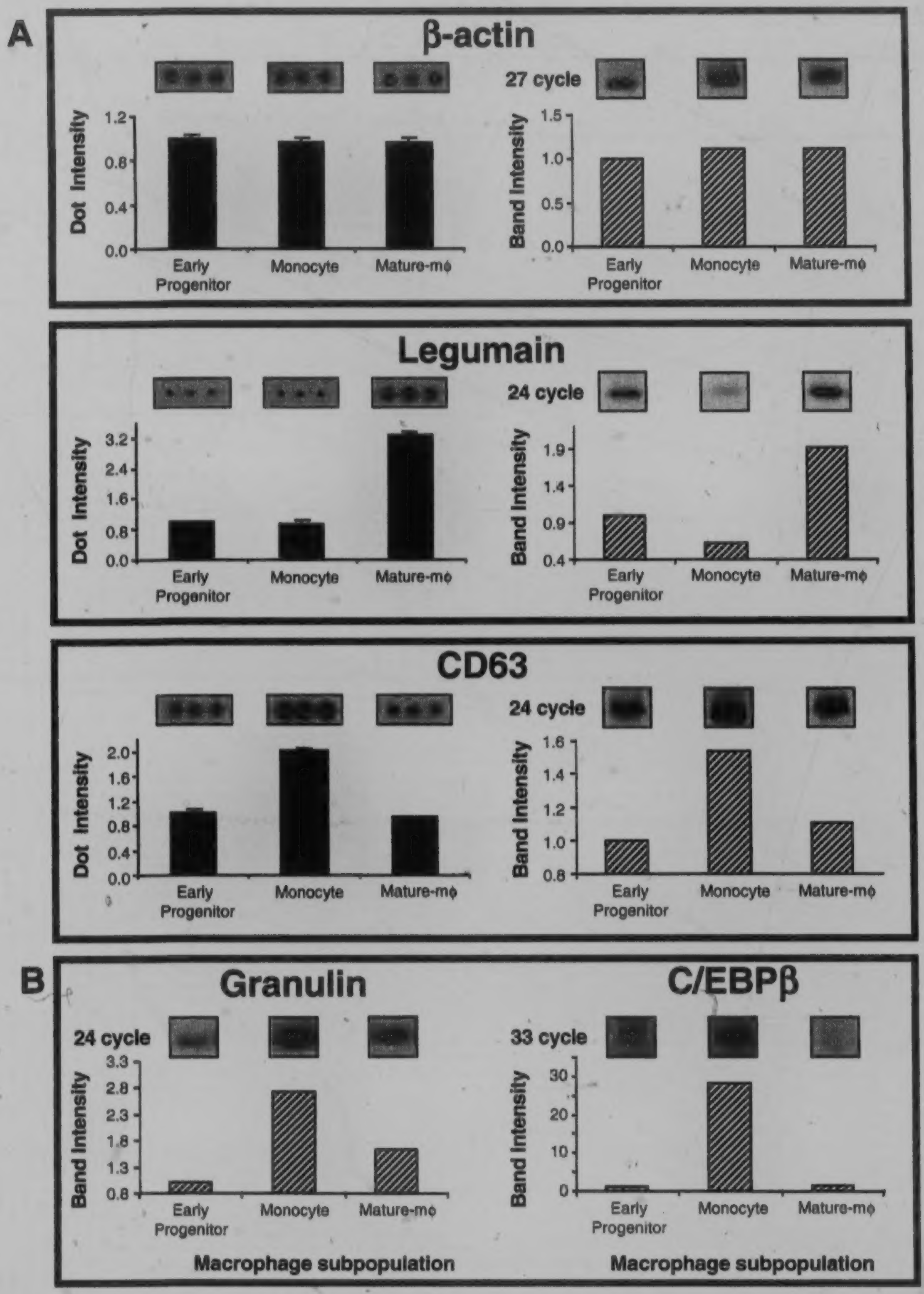


Figure 7.6: Characterization of potential markers of fish macrophage differentiation and/or function. Selected putative markers were chosen based on their relative level of differential expression among three stages of fish macrophage differentiation. Gene expression profiles are based on membrane array analysis results. Values are based on triplicate readings corresponding to each subset and were normalized to the early progenitor subpopulation (value=1) in order to determine the relative level of expression between the three stages of differentiation. Subsets are denoted by E.P: early progenitor, Mon: monocyte, Mϕ: mature macrophage. Transcript identities are based on results presented on Table 7.2.

Figure 7.7: Gene expression analysis of transcripts that displayed significant cross-reactivity with pro-mRNA species. For a number of transcripts the membrane array technique was unable to determine the differential levels of gene expression between early progenitor, monocyte, and "mature" macrophage subpopulations; this was due to the cross-hybridization of the gene-specific cDNA probe with complex pre-mRNA species and mRNA transcripts. In such cases we used semi-quantitative RT-PCR to examine transcript levels. A) Shows the correlation of gene expression profiles between membrane array and RT-PCR analyses for 3 transcripts readily detected using both techniques. B) Shows the subsequent analysis of 2 transcripts that required specific RT-PCR amplification. Each RT-PCR reaction was done in duplicate and examined at five stages of amplification to ensure comparisons were made during the linear portion of the amplification phase. RT-PCR values were subsequently normalized to the early progenitor subpopulation (value=1) to determine the relative level of expression between the three macrophage subpopulations; representative results are shown.


A
β-actin
27 cycle
Dot Intensity
0.0
0.4
0.8
1.2
Band Intensity
0.0
0.5
1.0
1.5
Early Progenitor
Monocyte
Mature-mϕ
Legumain
24 cycle
Dot Intensity
0.0
0.8
1.6
2.4
3.2
Band Intensity
0.4
0.9
1.4
1.9
CD63
24 cycle
Dot Intensity
0.0
0.4
0.8
1.2
1.6
2.0
Band Intensity
0.8
1.0
1.2
1.4
1.6
B
Granulin
24 cycle
Band Intensity
0.8
1.3
1.8
2.3
2.8
3.3
C/EBPβ
33 cycle
Band Intensity
0
10
20
30
Macrophage subpopulation

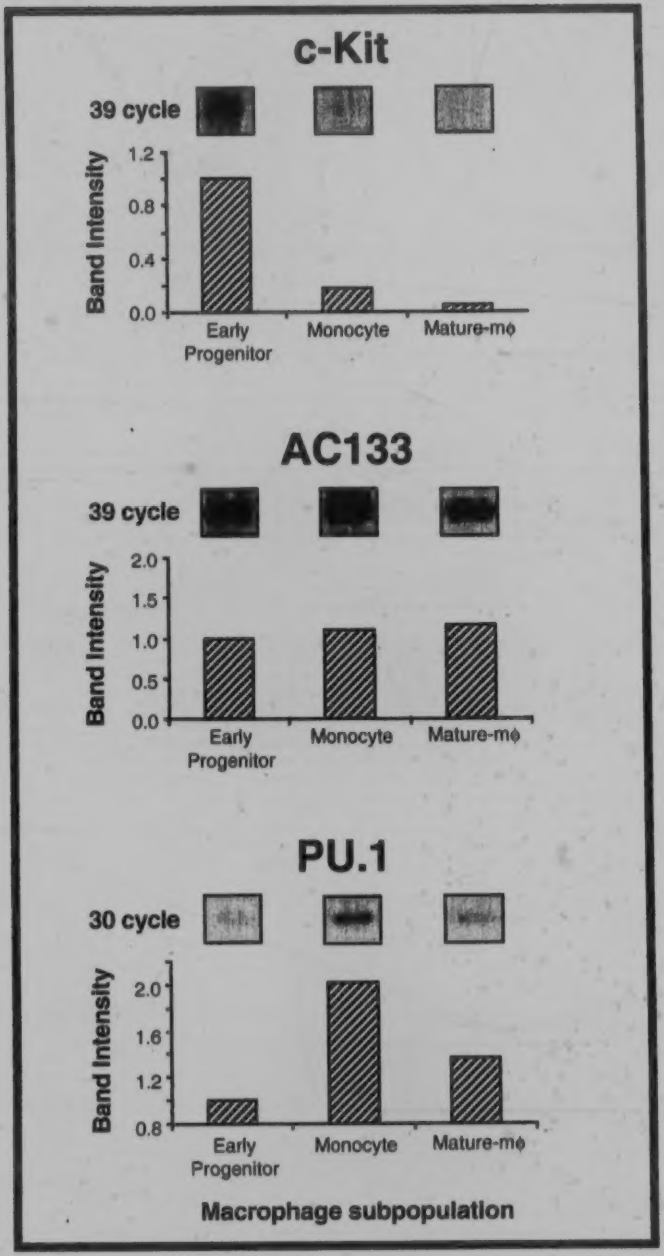


Figure 7.8: Gene expression analysis for additional markers of hematopoiesis. RT-PCR confirmed the patterns of gene expression for three known markers of hematopoiesis. Each RT-PCR reaction was done in duplicate and examined at five stages of amplification to ensure comparisons were made during the linear portion amplification phase. RT-PCR values were subsequently normalized to the early progenitor subpopulation (value=1) to determine the relative level of expression between the three macrophage subpopulations; representative results are shown.

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CHAPTER VIII: GENERAL DISCUSSION

1. OVERVIEW OF THE FINDINGS

Macrophages play a central role in the innate immune responses of lower vertebrates such as bony fishes. Maturation of early hemopoietic macrophage progenitors leads to the production of potent effector cells with important roles in homeostasis and antimicrobial host defenses that include, among others, phagocytosis and the production of bioreactive pathogen-killing molecules. Macrophages also display distinct functional characteristics at specific differentiation stages, tailored to the immunological demands and the environment where they reside. It is, therefore, essential to understand the mechanisms leading to the generation of functionally mature macrophages in fish, and the role that these cells play in host defense; this is necessary not only from an evolutionary perspective, but also for the design of prevention strategies against fish pathogens.

The main focus of my Ph.D. program was to characterize the soluble factors and the developmental pathways that regulate the production of functionally mature macrophages from early hemopoietic progenitors. Cultivation of goldfish kidney leukocytes in the presence of cell-conditioned supernatants from a goldfish macrophage cell line (GMCL) indicated that goldfish macrophages could be readily grown *in vitro* (Chapter III). Moreover, cell-conditioned medium (CCM) from these primary kidney macrophage (PKM) cultures could be used in the establishment of new PKM cultures. Through a series of experiments we determined that goldfish PKM developed *in vitro* in response to endogenous macrophage growth factor(s) (MGF) (Chapter III). The predominant cell in these cultures was characterized as the macrophage based on the following criteria: a) non-specific esterase staining; b) morphological similarity to the spontaneously growing GMCL, maintained in culture for over 7 years; c) phagocytosis of the bacterium, *A. salmonicida*; and d) production of reactive oxygen and nitrogen intermediates in response to macrophage activating factors and/or LPS. Primary kidney macrophage cultures, like GMCL, comprised of three distinct subpopulations of cells based on flow cytometric analysis of their size and internal complexity parameters.

Characterization of these subpopulations using morphological, cytochemical, and functional analyses indicated that they represented cells of the macrophage lineage, but at distinct junctures of differentiation: early progenitors, monocytes, and "mature" macrophages. Subsequent analysis of their gene expression profiles indicated that they also expressed several differentiation markers, previously characterized in mammalian macrophages (Chapter VII).

Flow cytometric analysis of developing PKM shed light on the proliferation and differentiation events, which led to the generation of mature goldfish macrophages from early hematopoietic progenitors (Chapter IV). My results showed that PKM cultures proliferated non-synchroneously, suggesting the presence of a temporal control mechanism(s) that regulated the proportion of early progenitors entering the path towards maturation. Furthermore, PKM early progenitors appeared to develop through two alternate pathways of differentiation: one consistent with the classical mononuclear phagocyte system (MPS) pathway of macrophage development (early progenitors → monocytes → mature macrophages); and an "alternate" pathway of macrophage development where mature macrophages appeared to quickly develop from early progenitors in the absence of the typical MPS-monocyte intermediate stage.

I found that the spontaneous growth of PKM in response to endogenous MGF was strictly controlled through the production of endogenous macrophage growth inhibitory factor(s) (MGIF). These included a novel soluble form of the CSF-1 receptor (sCSF-1R) that may inhibit PKM proliferation through a growth factor deprivation-mediated mechanism (Chapter VI). Interestingly, only the early progenitor and mature macrophage PKM subpopulations showed detectable mRNA expression of the sCSF-1R transcript; these were also the only two subpopulations that were capable of self-renewal in the absence of exogenous growth factors (Chapter IV). Thus, it appeared that the development of spontaneously growing PKM was controlled through an endogenous CSF-1/CSF-1R regulatory loop. The discovery of the sCSF-1R and other positive and negative regulators of fish macrophage development, through a differential cross-screen approach, highlighted the inherent complexity in the hematopoietic regulatory machinery of lower vertebrates. Moreover, their identification through this approach provided a more global understanding of macrophage biology as they were examined in the context of expression analysis for a broad spectrum of genes.

2. IDENTIFICATION OF EMBRYONIC-LIKE MACROPHAGES IN ADULT FISH HEMATOPOIETIC TISSUES: A MODEL FOR THE GENERATION OF MACROPHAGE HETEROGENEITY.

The current views on the generation of macrophages from hematopoietic progenitors are based on the mononuclear phagocyte system (MPS) theory proposed by van Furth and colleagues (1-4). This theory describes all macrophages as recent progeny of bone marrow-derived monocytes, whose precursors commit to the macrophage lineage following the common bipotent granulocyte-macrophage progenitor (CFU-GM) (5). Monocytes travel through the circulatory system to the sites in the body where they are needed for host defense/housekeeping functions and differentiate into tissue macrophages in response to local stimuli. Based on kinetic studies, these authors placed considerable emphasis on the view that the majority of cell division in this lineage is restricted to the earliest stages of differentiation. Recent evidence supports that myeloid progenitors derived from hematopoietic stem cells (HSC), including common myeloid progenitors (CMPs) and their progeny, the granulocyte-macrophage and megakaryocyte-erythrocyte progenitors, have limited or no-self renewing capacity (6, 7). Renewal of resident macrophages is attributed to the regular influx and subsequent maturation of blood monocytes, rather than local proliferation (3, 4, 8). However, in keeping with the redundancy typically exhibited by the immune system, it is surprising that the development of such a central player to immune defense mechanisms and homeostatic control would be solely dependent on a single MPS-based pathway. This is particularly intriguing in light of the tremendous heterogeneity observed in macrophage morphology, cytochemistry, gene expression, and function (9-16).

My studies have shown that goldfish primary kidney macrophages (PKM) possessed several unique features that distinguished them from the classical mammalian MPS macrophages (MPS-mϕs). Many of these features appeared to center around PKM developing through the alternate pathway of differentiation (AP-mϕs), originally described in Chapter IV. The presence of this pathway may be limited to fish systems.

However, scanning of the mammalian literature identified several macrophage populations that appeared distinct from MPS-mϕs. Interestingly, a number of the unique goldfish AP-mϕ features that distinguished them from MPS-mϕs were consistent with descriptions of embryonic macrophages. In the following sections I will discuss several features of goldfish PKM alternate pathway macrophages (AP-mϕs) in the context of similar descriptions in the embryonic macrophage literature. In addition, several descriptions of specific mammalian tissue macrophages point to the contribution of MPS-independent mechanisms in the replenishment of discrete macrophage populations in the adult. Overall, I believe that the alternate pathway of macrophage differentiation identified in goldfish PKM is conserved across evolution and contributes to the generation of spontaneously growing macrophage populations. Moreover, this pathway is also developmentally conserved from primitive hematopoiesis in the vertebrate embryo to adult hematopoiesis. Embryonic macrophages, whether in fish or mammals, may represent the earliest members of an MPS-independent differentiation program that contributes significantly to the generation of macrophage heterogeneity.

2.1. Primitive hematopoiesis

In the embryo, primitive macrophages appear shortly after primitive erythrocytes in the first wave of hematopoietic development. Primitive or embryonic hematopoiesis occurs extra-embryonically in the yolk sac, it is exceedingly rapid, and appears to ensure that sufficient oxygen is supplied to the developing embryo and that dying cells, resulting from rapid tissue turnover, are efficiently cleared out (17, 18). While embryonic hematopoiesis is active but beginning to decline, an independent wave of hematopoiesis, termed definitive, is initiated within the embryo proper (17-19). Definitive hematopoiesis sustains production of all blood cell lineages for the fetus as well as the adult. Definitive hematopoiesis originates in the aorta-gonad-mesonephros (AGM) region of the para-aortic splanchnopleura (P-Sp), where *de novo* formation of hematopoietic stem cells (HSC) is believed to occur, followed by their migration from the AGM to the fetal liver, spleen, and the bone marrow where subsequent maturation takes place (17, 20-24).

As early as 1908, Dantschakoff showed that macrophages existed in the yolk sac during early ontogeny, before the initiation of bone marrow hematopoiesis (25). In the embryo, primitive macrophages expressing CSF-1R mRNA are already scattered throughout the body prior to the appearance of monocytes and granulocytes, and are retained in various embryonic tissues as resident macrophages (5, 9, 26). Embryonic macrophages appear to bypass the intermediate MPS-monocyte differentiation stage, and possess proliferative capacity [reviewed in (27, 28)]. Moreover, embryonic macrophages appear to develop through PU.1-independent mechanisms, do not show typical strong peroxidase activity, and do not express mature macrophage markers such as F4/80 (5, 9, 28-31).

The segregation of the two waves of macrophage production is retained across species. Recent observations indicate that the ontogeny of macrophages in early avian, mouse, and human embryos is different from those arising during definitive hematopoiesis and MPS-mediated differentiation (5). Similarly, in *Xenopus* and zebrafish primitive macrophages appear to arise from areas distinct from the typical hematopoietic hotspots (5). Interestingly, a homologue for the mammalian ABC1 transporter, expressed on embryonic macrophages and required for the engulfment of apoptotic bodies, has been identified as ced-7 in *C. elegans* (32). Moreover, the phenotype of ced-7 mutants is precisely defined by an impaired engulfment of cell corpses. Therefore, it appears that primitive macrophages have been conserved through evolution and serve, at least, to clear dying cells derived from the extensive tissue turnover that occurs during development.

2.2. Goldfish alternate pathway macrophages versus embryonic macrophages

Morphological, cytochemical, and functional analyses characterized cells in our *in vitro* model system as macrophages. These cells were recognized and infected by *Leishmania major*, an obligate intracellular parasite of macrophages. Moreover, they expressed several macrophage markers including the CSF-1 receptor gene, c-*fms*, presumably the most universal macrophage marker known so far, both across the heterogeneous populations of humans and mice and across species, from fish to humans (31, 33-35). Goldfish primary kidney macrophages (PKM) developed through two alternate pathways of differentiation. It was the characterization of goldfish alternate pathway macrophages (AP-mϕs) that suggested that embryonic macrophages and/or their precursors may be retained in the adult fish. AP-mϕs, originally referred to as R2-type macrophages in Chapter III, appeared quickly and generally preceded the appearance of MPS-like monocytes (Chapter III). AP-mϕs, like embryonic macrophages, and in stark contrast to MPS-mϕs, were also capable of self-renewal (Chapter IV). Consistent with the peroxidase activity of embryonic macrophages and some resident steady-state macrophages [reviewed in (29, 36)], we found that AP-mϕs displayed localized myeloperoxidase staining in and around the nuclear envelope (Chapter III). This was in sharp contrast to the MPS-like monocyte subpopulation (R3-type macrophages) that showed the typical cytoplasmic peroxidase activity of mammalian monocytes and exudate monocyte-derived macrophages.

The expression of PU.1/spi-1 in primary kidney macrophage subpopulations (Chapter VII) also pointed to the parallels between goldfish AP-mϕs and embryonic macrophages. PU.1, a member of the Ets transcription factor family, is a critical regulator of hematopoiesis (37). In particular, its early expression during hematopoiesis is essential for the positive regulation of myeloid and B-lymphoid development, and the negative regulation of commitment towards the erythroid lineage [reviewed in (38)]. In adult MPS-mϕ, PU.1 is expressed at high levels (39). In fact, PU.1 is absolutely required for macrophage development after commitment to the MPS pathway (40). This is consistent with the numerous macrophage promoters that have functionally essential PU.1 sites. These include, for example: CSF-1, GM-CSF, and G-CSF receptors (41-43); lysozyme (44); myeloperoxidase (45); Fc receptors (FcRI and FC RIIIA) (46-48); CD11b (49, 50); and Toll-like receptor 4 (51). Surprisingly, PU.1 does not appear to be required for embryonic macrophage development, presumably due to functional redundancy with other transcription factors such as MITF, Spi-B, or members of the c/EBP family (31, 52). However, there are mixed reports as to its expression; in mouse embryo PU.1 expression was not detected until the onset of liver hematopoiesis despite the presence of *c-fms* positive phagocytes early in embryonic development (31). More recent studies in zebrafish, however, indicated that spi-1/PU.1 expression was first detected at 12 hours post-fertilization, where it co-localized with expression of other early macrophage markers and preceded c-*fms* expression (37, 53). Our results indicated that AP-mφs like embryonic mφs expressed PU.1, but at significantly lower levels than cells differentiating along the MPS pathway (Chapter VII).

2.3. Other MPS-independent macrophage populations

In recent years it has become apparent that hematopoietic progenitors with tremendous plasticity can be isolated from tissues outside the bone marrow (18, 54-58). Whether these progenitors are derived from migrating HSC, or are a result of trans-differentiation or de-differentiation events, their identification draws attention to non-classical mechanisms of blood cell formation. Macrophages are no exception; tissue-specific macrophage precursors and discrete populations of self-renewing macrophages have been described (39, 59-62). Early observations were based on, for example, the elimination of circulating monocytes by Strontium-89 treatment, which did not affect the total numbers or proliferation potential of resident macrophage populations in the peritoneum, lungs, or liver (8, 63, 64). This is consistent with recent transplantation experiments, where the reconstitution potential of tissue macrophage populations by lacZ-expressing bone marrow donor cells was examined. Briefly, whereas the majority of bone marrow and splenic macrophages became of donor origin within one month, only 61% of lung and liver macrophages appeared to be replaced by donor cells after one year, and microglia were even less likely to be turned over (28, 65, 66).

Microglia-based studies provide further insights into MPS-independent mechanisms of macrophage development. Microglia-specific progenitor cells were identified within the brain, a site not readily accessible for re-population via influx of monocytes due to the blood-brain barrier (59). More recently, a closer analysis of the origin of these macrophage populations indicated that they appeared to be derived from progenitor cells that arose in the yolk sac during embryonic development (67). Similarly, observation of embryonic lungs indicated that macrophages developed quickly through a burst in cellular divisions unlike those described for infiltrating MPS-monocytes, and that these macrophages appeared prior to the establishment of the bone marrow and the appearance of monocytes in the circulation (61). It also was found that stimulation of rat fetal lung macrophage precursors with M-CSF, GM-CSF, G-CSF, or IL-3 yielded in all cases macrophage cultures, which suggested that these precursors were already committed to the macrophage line (61). Interestingly, these mechanisms of CSF stimulation may not be restricted to *in vitro* conditions, as both M-CSF and GM-CSF are normally produced in postnatal lung tissues (61, 68, 69).

2.4. Goldfish alternate pathway macrophages (AP-mϕs) and the endogenous control of macrophage growth and function/activation.

In the sections above, I have discussed several parallels between embryonic macrophages, goldfish AP-mϕs, and various adult macrophage populations that appear to develop through MPS-independent mechanisms. Goldfish AP-mϕs, however, also displayed a number of novel features that could potentially contribute to the growth and function of MPS-independent macrophage populations, and may facilitate their future characterization.

AP-mϕs proliferated in response to endogenous macrophage growth factors (MGF; Chapter III and IV). Both the early progenitor and "mature" macrophage PKM subsets were capable of proliferation in the absence of exogenous growth factors. In contrast, sorted MPS-like monocytes remained in and around their FACS-denoted R3 gate and had decreased viability when cultured in growth medium only (Chapter IV). This was consistent with the mammalian monocytes that required exogenous cytokines such as CSF-1, IL-3, and IL-6 for their survival and differentiation into mature macrophages (70-74). The native primary kidney macrophage endogenous MGF was not isolated. However, characterization of partially purified supernatants and PKM phenotype suggested the presence of a CSF-1-like molecule in goldfish PKM supernatants. In particular, this was supported by the identification of a soluble CSF-1R-mediated temporally-regulated control mechanism of macrophage growth in goldfish PKM (Chapter VI).

Several studies point to local growth factor production in tissues where MPS-independent macrophages reside (23, 61, 68). It would be interesting to determine if such populations produce endogenous macrophage growth inhibitory factors (MGIF), including soluble forms of the CSF-1R, that may establish local regulatory loops of macrophage development. Characterization of genomic sequences for the c-*fms* gene

(Chapter VI) indicated the potential for alternatively spliced soluble forms of the CSF-1R across species, thus establishing the potential for the presence of such regulatory negative feedback mechanisms.

Zebrafish yolk sac-derived embryonic macrophages have been shown to effectively eliminate bacterial infections before the appearance of any other leukocytes (75). More recently, Herbomel and colleagues demonstrated that macrophages arose early in zebrafish development and appeared to originate through mechanisms distinct from those of monocytic cells (34, 76). These macrophages were also shown to have phagocytic activity and were able to eradicate both gram-positive and gram-negative bacterial challenges. The killing responses of these primitive macrophages, however, were not thoroughly examined. If embryonic macrophages prove to develop through the same differentiation program that led to the development of AP-mϕs in goldfish PKM cultures, future experiments may find additional parallels in their functional responses. In particular, it would be interesting to determine if the killing responses of these embryonic macrophages are limited to phagocytosis, or include strong nitric oxide responses similar to those displayed by AP-mϕs in PKM cultures (R2-mature macrophages in Chapter III). Experiments in our laboratory have already pointed to endogenous mechanisms of activation of AP-mϕs in response to transferrin proteolytically-cleaved products, which induced the production of reactive nitrogen intermediates (77, 78). The transferrin cleavage process was mediated by stimulated goldfish kidney leukocytes and products released from damaged/lysed goldfish leukocytes. One could reason that transferrin would allow for activation of embryonic macrophages in the absence of the classical activation cytokines like lymphocyte-derived IFNγ or IL-2, likely absent early in development prior to the definitive wave of hematopoietic development. Transferrin is a ubiquitously distributed endogenous molecule even early in embryonic development (79-84), and is secreted by activated immune cells such as macrophages (77). Therefore, transferrin may provide an efficient antimicrobial mechanism early in development in the absence of the classical activation cytokines and additional killing leukocytes, as it would be readily available at sites of infection, and be cleaved by products released at the site of inflammation.

The isolation and characterization of several differentially expressed genes in primary kidney macrophages (PKM) in Chapter VII identified two patterns of gene expression that may be useful for the future characterization of alternate pathways of macrophage differentiation. The first one, illustrated by PU.1, C/EBPβ, CD63, granulin, IFN-inducible protein, and nephrosin, showed preferential gene expression in the MPS-like monocyte stage. I believe that this was associated with the two alternate differentiation pathways identified in PKM cultures: a progressive increase in gene expression as cells migrated through the classical MPS-like pathway (early progenitors → monocytes); and consistent/decreased expression as cells migrated though the alternate pathway of development (early progenitors → mature macrophages). Although this profile may indicate stage-specific up-regulation of gene expression, the second pattern of gene expression points more strongly to the hypothesis above. The soluble form of the CSF-1R, 14-3-3 zeta, and to a lesser extent legumain show preferential expression in the early progenitor and mature macrophage stages. Although their expression has not been characterized across various embryonic and adult macrophage populations, examination of these genes may lead to the identification of markers for macrophages differentiating along MPS-independent pathways, and may point to phenotypic and/or functional differences between macrophages derived from classical and MPS-independent programs.

It remains unclear whether embryonic macrophage precursors retain long-term reconstitution potential. However, in light of my results it is tempting to speculate that AP-mϕs may represent a population derived from embryonic macrophage precursors that may have remained into adulthood and contribute to the generation of macrophage diversity in the adult fish. Furthermore, the presence of similar discrete populations in mammals that appear to develop through monocyte-independent pathways may point to the evolutionarily conservation of such MPS-independent mechanisms of macrophage differentiation in the adult. I predict that future analysis of macrophage hematopoietic pathways will show a compartmentalization of the contribution of these pathways to the various discrete macrophage populations throughout the vertebrate adult. It will be interesting to determine the basis for the hematopoietic control of alternate pathways of macrophage development, the distribution of these macrophages throughout the vertebrate body, and the differential roles that macrophages derived from each of these pathways play in host defense and the maintenance of homeostasis.

3. FUTURE DIRECTIONS

The results presented in this thesis indicate that fish myelopoiesis, as previously described in mammals, is an inherently complex process under stringent regulation by an array of intrinsic and extrinsic modulators. The *in vitro* primary kidney macrophage model characterized in my studies provided a great vehicle into the control mechanisms of goldfish macrophage development. Part of the attractiveness of this model system was derived from the largely sit-back, observation-based approach that could be taken when examining PKM growth. *In vitro*-derived PKM developed preferentially from a pool of hematopoietic progenitors as a result of endogenous macrophage growth factor production; this minimized much of the complexities associated with attempting to study regulatory events in the presence of various cell types. Also unique was the opportunity to examine the progression of macrophages through their hematopoietic pathways *in vitro*; not only does this warrant further characterization of the mechanisms modulating the progression from early progenitors to mature macrophages, but also examination of the interactions between macrophages at different stages of maturation.

Although much has been learned from the mechanisms controlling macrophage development in the goldfish, a major constraint has been our inability to obtain pure preparations of fish cytokines. My research will facilitate the future isolation of cytokines, such as a CSF-1-like molecule, from PKM supernatants. I have characterized the phases at which PKM likely produce the majority of growth promoting and growth inhibitory factors (*proliferative* and *senescence* PKM phases, respectively), have established protocols for their collection, and have cloned and expressed a soluble form of the CSF-1 receptor. Recombinant sCSF-1R will be subsequently used in the isolation of native fish CSF-1.

Functional studies attempting to address the role of goldfish CSF-1 in the induction of macrophage survival, proliferation, differentiation, and function will require significant amounts of CSF-1, unlikely to be purified in sufficient amounts in its native form. As a result, future studies will require the cloning and expression of goldfish CSF-

1 in order to facilitate its characterization. Following characterization and comparison of native and recombinant goldfish CSF-1, researchers will be able to assess its contribution to the generation of PKM through the two pathways of macrophage development. Because of the various unique features of the alternate pathway, I predict that this pathway will become increasingly interesting. Moreover, there is a need to examine the mechanisms leading to CSF-1 and sCSF-1R production in the context of the three phases of macrophage development described in Chapter V. This will provide a further understanding of the predicted CSF-1/CSF-1R endogenous regulatory loop.

The contribution of CSF-1 to the primary kidney macrophage MPS-like pathway is presently unknown. However, I anticipate that even if CSF-1 does play an essential role in their development, additional factors will be required for the differentiation of early progenitors along this pathway. To this end we are currently expressing additional positive modulators of hematopoiesis that were identified through the differential cross-screening technique. In addition, characterization of fish cytokines such as IL-6 and TNF-α is currently in progress in our laboratory.

The main objectives of my thesis were to examine the development of goldfish macrophages *in vitro*, and to identify positive and negative modulators of fish macrophage hematopoiesis. My research has provided the first step in our goal to characterize the mechanisms involved in the regulation of fish macrophage development. The understanding of fish macrophage hematopoiesis will facilitate the design of prevention strategies against fish pathogens and provide an improved understanding of the evolutionary conservation of macrophage-derived defense mechanisms and their overall function.

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